UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

André Luis Giacometti Conceição

Análise da expressão e de mecanismos de regulação de genes envolvidos na transição epitélio mesênquima em carcinoma renal de células claras

São José do Rio Preto
2016

André Luis Giacometti Conceição

Análise da expressão e de mecanismos de regulação de genes envolvidos na transição epitélio mesênquima em carcinoma renal de células claras

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientadora: Profª. Drª. Paula Rahal

São José do Rio Preto
2016

Conceição, André Luis Giacometti.
Análise da expressão e de mecanismos de regulação de genes envolvidos na transição epitélio mesênquima em carcinoma renal de células claras / André Luis Giacometti Conceição. -- São José do Rio Preto, 2016
77 f. : il., tabs.

Orientador: Paula Rahal
Tese (doutorado) – Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências, Letras e Ciências Exatas

1. Genética. 2. Expressão gênica. 3. Carcinoma de células renais. 4. Epigenética. I. Rahal, Paula. II. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. III. Título.

CDU – 575.113

Ficha catalográfica elaborada pela Biblioteca do IBILCE
UNESP - Câmpus de São José do Rio Preto

André Luis Giacometti Conceição

Análise da expressão e de mecanismos de regulação de genes envolvidos na transição epitélio mesênquima em carcinoma renal de células claras

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientadora: Profª. Drª. Paula Rahal

Comissão Examinadora

Drª. Marilia de Freitas Calmon Saiki
UNESP – São José do Rio Preto
Co-orientadora

Prof. Dr. Wilson Araújo da Silva Junior
USP – Ribeirão Preto

Profª. Drª. Aparecida Maria Fontes
USP – Ribeirão Preto

Profª. Drª. Patricia Simone Leite Vilamaior
UNESP – São José do Rio Preto

Profª. Drª. Flávia Cristina Rodrigues Lisoni
UNESP – Ilha Solteira

São José do Rio Preto
16 de fevereiro de 2016

RESUMO

O carcinoma renal de células claras (ccRCC) é o mais comum dos subtipos histológicos de carcinoma renal. Este carcinoma é histologicamente caracterizado pela presença de células com citoplasma claro e abundante. O processo inicial de metástase pode ser atribuída à transição epitélio mesênquima (EMT). Neste estudo, buscou-se identificar genes diferencialmente expressos em ccRCC, construindo assim um perfil molecular para este tumor. Nós selecionamos genes descritos na literatura que apresentam relação com EMT, diferenciação e proliferação celular. Analisou-se por PCR quantitativo e imuno-histoquímica a expressão dos genes e suas proteínas, respectivamente, e os possíveis mecanismos epigenético que regulam a sua expressão em amostras de ccRCC e linhagem celular. Os genes *OCLN* e *GAS1* foram encontrados com baixa expressão em ccRCC, e nós sugerimos que o miR-122 e miR-34a podem regular sua expressão, respectivamente, neste tipo de câncer. Além disso, mostramos, por qPCR e imuno-histoquímica, a alta expressão de SLC2A1 foi significantemente alta em ccRCC. O conjunto de genes identificados neste estudo possibilita a compreensão das bases moleculares e de desenvolvimento do ccRCC.

Palavras-chave: Carcinoma renal de células claras. Expressão gênica. Epigenética.

ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of kidney cancer. This carcinoma is histologically characterized by the presence of clear and abundant cytoplasm. The initial process of ccRCC metastatic tumor formation can be attributed to epithelial-mesenchymal transition (EMT). In this study, we sought to identify genes differentially expressed in ccRCC and build a molecular profile of this cancer. We selected genes described in the literature to be related to EMT, cellular differentiation and proliferation. We analyzed the gene and protein expression by quantitative PCR and immunohistochemistry, respectively, and examined possible epigenetic mechanisms that regulate their expression in ccRCC samples and cell lines. OCLN and GAS1 genes were under-expressed in ccRCC, and we report that miR-122 and miR-34a, respectively, may regulate their expression in this cancer. Furthermore, we showed by quantitative PCR (qPCR) and immunohistochemistry that SLC2A1 was significantly over-expressed in ccRCC. The set of genes identified in this study furthers our understanding of the molecular basis and development of ccRCC.

Keywords: Clear cell renal cell carcinoma. Gene expression. Epigenetics

Ao meu amado e misericordioso Deus...

Por que parece ser tão mais fácil reclamar da vida? Por que temos uma certa tendência a ficarmos insatisfeitos e a achar que estamos muito longe de tudo aquilo que sonhamos. Por que a grama do vizinho parece ser tão mais verde do que a nossa? Será que é tão difícil olhar para a nossa vida e para nós com o mesmo olhar que lançamos para os outros?

Nós precisamos aprender a ter orgulho do que somos, do que fazemos e do que temos. Precisamos ser menos críticos e menos cruéis conosco, precisamos saber relevar as nossas falhas e fraquezas e saber celebrar as pequenas conquistas diárias. Os nossos dias são feitos de pequenos passos e é com o sucesso dos pequenos passos que vamos chegar onde queremos.

É com este olhar generoso sobre mim e sobre a minha vida que eu dedico e agradeço a Deus tudo o que eu alcancei até hoje. Eu reconheço o meu esforço e me reconcilio com os meus fracassos, porque sei que nenhum deles foi grande o suficiente para me fazer desistir. Pelo contrário, os meus erros me ajudaram a crescer.

Muito obrigado, meu Deus, por tudo de bom e de mau que é colocado no meu caminho, pois aquilo que não me traz felicidade me permite reconhecê-la quando ela chega em minha vida.

Ao meu grande amor, Bruna Stuqui,

pela companheira fundamental e brilhante que você foi, é e tenho certeza que sempre será. Obrigado por estar ao meu lado diante dessa nova conquista. Durante esses 5 anos do meu doutorado passamos por momentos que ficarão marcados na nossa história eternamente. O principal deles foi o tão sonhado e esperado casamento. Posso afirmar que foi a melhor escolha feita na minha vida.

Como a vida não é o um mar de rosas passamos por alguns momentos difíceis, mas graças a Deus e ao nosso amor, permanecemos sempre unidos.

Queria te agradecer por tudo que passamos, sem você nada disso teria acontecido, obrigado pela presença em minha vida. Te amo...

À Profª. Drª. Paula Rahal

pela pessoa ímpar como profissional dedicada, orientadora extremosa, obrigado por sua honradez e pelo seu incentivo, pela oportunidade que me foi dada. Agradeço com imenso respeito e admiração e por todos os momentos de paciência, puxões de orelha e preocupação.

Aos meus pais Juçara e Angelo,

os mais valiosos seres humanos com quem convivo desde sempre. Devo a eles tudo que eu sou em todos os aspectos. Não teria chegado a esse momento tão importante e significativo de minha vida, se eles não fossem pessoas corajosas, fortes e generosas que sempre foram. Eles continuam sendo a minha maior referência.

AGRADECIMENTOS

À Drª Marilia de Freitas Calmon, a quem devo grande parte da minha evolução enquanto aprendiz de pesquisador. Sem sua contribuição, tudo seria mais difícil.

À Drª Miriam, Drª Camila e à Jessica pela colaboração no desenvolvimento de algumas técnicas e pela excelente recepção no Laboratório de Ontogenia e Epigenética da Universidade Federal de São Paulo (UNIFESP).

À Drª Marina e Rodolfo pela colaboração e, principalmente pela amizade e apoio durante todo esse tempo.

Aos amigos do Laboratório de Estudos Genômicos do Instituto de Biociências, Letras e Ciências Exatas, Ana Beatriz, Ana Claudia, Bruno, Carina, Caroline, Cintia, Guilherme, Jacqueline, Lenira, Leticia, Maria Leticia, Mariana, Marília, Marina Dias, Monica, Natalia, Paola, Renata, Rafael, Stephane, uma equipe a qual é exemplo de trabalho com alegria e entusiasmo.

À Universidade Estadual Paulista – Instituto de Biociências, Letras e Ciências Exatas (UNESP-IBILCE) pela oportunidade de adquirir conhecimentos. Jamais esquecerei as aulas, cursos e tudo. Levarei a referência de qualidade da UNESP-IBILCE aonde chegarem meus passos.

Os meus sinceros agradecimentos aos meus sogros Elisabete e Rubens, com os quais sempre aprendi a importância da solidariedade e do amor, agradeço por me ajudarem, tantas vezes, na execução das tarefas cotidianas.

Aos meus amigos Ana Carolina, Ana Luiza, André, Camila, Eduarda, George, Juliana, Larissa, Marcela, Mariana, Rafael e demais, companheiros de todos os momentos e que sempre torceram pelo meu crescimento.

À CNPq e FAPESP pela bolsa e apoio financeiro ao laboratório.

LISTA DE ABREVIAÇÕES E SIGLAS

bp	Pares de bases, do inglês *Base pairs*
BSA	Soroalbumina bovina, do inglês *Bovine* serum *albumin*
ccRCC	Carcinoma renal de células claras, do inglês *Clear cell renal cell carcinoma*
CDH1	*Caderina 1* ou *E-Caderina*, do inglês *Epithelial cadherin*
CDH2	*Caderina 2* ou *N-Caderina*, do inglês *Neuronal cadherin*
cDNA	DNA complementar, do inglês *Complementary DNA*
ChiP	Imunoprecipitação da cromatina, do inglês *Chromatin immunoprecipitation*
CLDN1	*Claudina 1*, do inglês *Claudin 1*
CpG	Citosina – fosfato - Guanina
CTSB	*Catepsina B*, do inglês *Cathepsin B*
DAB	Solução 3,3'-diaminobenzidina
Dicer	Ribonuclease III Dicer
DMEM	do inglês, *Dulbecco's modified eagle medium*
DMSO	Dimetilsulfóxido
DNA	Ácido Desoxirribonucleico, do inglês *Deoxyribonucleic acid*
DNMT1	DNA metiltransferase 1
DNMT3A	DNA metiltransferase 3 alpha
DNMT3B	DNA metiltransferase 3 beta
DNMT3L	DNA metiltransferase 3-like
Drosha	Ribonuclease III Drosha
EMT	Transição Epitélio Mesênquima, do inglês *Epithelial-Mesenchymal transition*
FHIT	Tríade frágil de histidina, do inglês *Fragile histidine triad*
GAPDH	Gliceraldeído-3-fosfato dehidrogenase
GAS1	do inglês *Growth arrest-specific1*
HCV	Vírus da Hepatite C, do inglês *Hepatitis C* virus
HDAC2	Deacetilase de Histona 2, do inglês *Histone deacetylase 2*
H&E	Hematoxilina e Eosina

HIF1α	*Fator indutor de hipóxia 1α*, do inglês *Hypoxia inducible factor 1α*
HIF2α	*Fator indutor de hipóxia 2α*, do inglês *Hypoxia inducible factor 2α*
H3K4me3	Trimetilação da Lisina 4 na Histona 3
H3K27me3	Trimetilação da Lisina 27 na Histona 3
H3K4me2	Dimetilação da Lisina 4 na Histona 3
H3K9me2	Dimetilação da Lisina 9 na Histona 3
H3K9ac	Acetilação da Lisina 9 na Histona 3
H3K18ac	Acetilação da Lisina 18 na Histona 3
IgG	Imunoglobulina G
LAMβ1	*Laminina β1*, do inglês *Laminin β1*
mg	Miligrama
miR-34	microRNA 34
miR-122	microRNA 122
ml	Mililitro
mm	Milímetro
mRNA	Ácido ribonucleico mensageiro, do inglês *Messenger ribonucleic acid*
miRNA	microRNA
MTT	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina
ng	Nanograma
nM	Nanomolar
nm	nanômetro
OCLN	*Ocludina,* do inglês *Occludin*
PAFAH2	*Fator de ativação plaquetária acetilhidrolase 2*, do inglês *Platelet-activating factor acetylhydrolase 2*
PHGDH	*Fosfoglicerato Desidrogenase*, do inglês *Phosphoglycerate dehydrogenase*
PCR	Reação em cadeia da polymerase, do inglês P*olymerase chain reaction*
pH	Potencial hidrogeniônico
Pre-miR	microRNA precursor
Pri-miR	microRNA primário
qPCR	PCR em tempo real ou PCR quantitativo, do inglês *Quantitative PCR*
RISC	Complexo de silenciamento induzido por RNA, do inglês *RNA-induced silencing complex*

RNA	Ácido ribonucleico, do inglês *Ribonucleic acid*
RT-qPCR	PCR com transcriptase reversa seguido de PCR quantitativo, do inglês *Quatitative reverse transcription PCR*
SAM	S-adenosilmetionina
Snail	do inglês *Snail family zinc finger*
SLC2A1	*Carreador de soluto família 2 membro 1*, do inglês *Solute carrier family 2 member 1*
TGF-β1	*Fator de transformação do crescimento β1*, do inglês *Transforming growth factor β1*
TJ	Junção *tight*, do inglês *Tight junction*
TJP1	*Proteína da zona ocludente 1*, do inglês *Tight junction protein 1*
TSA	Tricostatina A, do inglês *Trichostatin A*
TSS	Sítio de Iniciação de Transcrição, do inglês *Transcription start site*
U	Unidade, do inglês *Unit*
UTR	Região não traduzida do RNA, do inglês *Untranslated region*
U48	Sinônimo de SNORD48, do inglês *Small nucleolar RNA, C/D box 48*
VEGF	Fator de crescimento endotelial vascular, do inglês *Vascular endothelial growth factor precursor*
VHL	*Ubiquitina ligase E3*, do inglês *von Hippel-Lindau tumor suppressor*
VIM	*Vimentina*, do inglês *Vimentin*
Zeb	do inglês *Zinc finger E-box*
5-Aza-dC	do inglês *5-Aza-2'-deoxycytidine*
5meC	5-metilcitosina
µg	Micrograma
µM	Micromolar
µm	Micrometro

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

INTRODUÇÃO

INTRODUÇÃO

A tumorigênese é um processo de acúmulo de anormalidades genéticas e epigenéticas que levam à disfunção celular. Os tumores apresentam uma grande heterogeneidade em sua morfologia celular, índice proliferativo, lesões genéticas e resposta terapêutica (VISVADER, 2011). No desenvolvimento do câncer, as células adquirem uma organização atípica, não expressando o mesmo conjunto de genes que suas contrapartes normais, sendo tal diferença a marca do estado canceroso (COOPER, 2000). A formação de um tumor pode resultar de uma proliferação anormal de qualquer tipo diferente de célula do corpo, possuindo diferentes velocidades de crescimento e capacidade de invadir outros tecidos e órgãos (INCA, 2015; COOPER, 2000).

O tumor formado nas neoplasias benignas é uma massa localizada de células que se multiplica vagarosamente assemelhando-se ao tecido original (INCA, 2015), e permanecem confinadas em seu local de origem, não invadindo os tecidos normais que o rodeiam e não difundindo para partes distantes do corpo (COOPER, 2000). Isso faz com esse tipo de tumor represente um pequeno risco, por apresentarem a mesma função que as células normais. Entretanto os tumores benignos podem tornar-se um problema sério, quando eles interferem nas funções teciduais normais, ou caso eles secretem quantidades excessivas de substâncias biologicamente ativas (LODISH, 2000). Em contrapartida, as neoplasias malignas apresentam um crescimento desordenado de células podendo invadir tecidos e órgãos. Os diferentes tipos de câncer correspondem aos diversos tipos de células do corpo, podendo ser distinguidos pela sua velocidade de multiplicação e capacidade de sofrer metástase (INCA, 2016).

O carcinoma de células renais é o décimo segundo tipo de câncer mais comum no mundo, com aproximadamente 338000 novos casos diagnosticados em 2012 (WCRFI, 2016). As estimativas mais recentes da *American Cancer Society* para esse tipo de câncer nos Estados Unidos em 2015 são de cerca de 61560 de novos casos (38270 em homens e 23290 em mulheres) (ACS, 2016). Dados publicados pela *International Agency for Research on Cancer* apontam que no Brasil mais de 4000 pessoas recebem o diagnóstico de câncer de células renais anualmente, sendo que desta população 61,7% são homens e para cada dois pacientes que são diagnosticados, um já com diagnóstico da doença vem a falecer (IARC, 2016).

A determinação dos diversos subtipos histológicos dos carcinomas de células renais apresenta implicação direta na escolha terapêutica a ser utilizada (LOPEZ-BELTRAN et al., 2006). O tumor renal pode ser classificado em diferentes subtipos histológicos, incluindo carcinoma renal de células claras, papilífero, cromófobo, cístico-sólido, ductos coletores de Bellini, medular, translocação Xp11, tubulomucinoso e células fusiformes, associado a neuroblastoma e não classificados (LOPEZ-BELTRAN et al., 2009). O carcinoma renal de células claras, o papilífero e o cromófobo compreendem 90% de todos os carcinomas de células renais (LOPEZ-BELTRAN et al., 2009). O carcinoma cromófobo tem sido relatado em 5% dos casos de câncer renal, apresenta células grandes com citoplasma reticulado. Os carcinomas papilíferos representam cerca de 10% dos carcinomas renais, são morfologicamente caracterizados pela presença de papilas contendo núcleo com agregados de macrófagos, grânulos de hemossiderina e cálcio (NELSON; EVANS; LARA, 2007; LABER, 2006).

O carcinoma renal de células claras (ccRCC) é o subtipo histológico mais comum de câncer renal em adultos, cerca de 70 – 75% dos casos (SHUCH et al., 2015). Este carcinoma tem origem no epitéio tubular do néfron proximal e é caracterizado histologicamente pela presença de células claras e com citoplasma abundante (KURODA et al., 2011; LOPEZ-BELTRAN et al., 2006; COHEN et al., 2005). Aproximadamente 30% dos pacientes em estágios iniciais da doença desenvolvem metástase após a cirurgia (COHEN et al., 2005). As metástases nos ccRCC comprometem principalmente pulmão, fígado e ossos (SANDRASEGARAN et al., 2010). As características agressivas do ccRCC interferem na eficiência de abordagens de tratamento convencionais, tais como quimioteraria e radioterapia (NAGATA et al., 2012).

Embora a quimioterapia e a radioterapia possam ser utilizadas, o tratamento do carcinoma renal ocorre principalmente por meio da nefrectomia parcial ou total e a imunoterapia com interferon-α e interleucina-2, as quais são modalidades terapêuticas bem estabelecidas (DRUCKER, 2005). No entanto, 30% dos pacientes tratados desenvolvem metástases, apresentando uma expectativa de vida de 12 meses (PETRELLA; BRINCKEHOFF, 2006; DRUCKER, 2005; TAKAHASHI et al., 2001).

Diversos fatores de risco estão associados com a incidência desta doença, dentre eles o consumo de tabaco e álcool, obesidade, hipertensão, fatores hormonais e

predisposição genética (ZBAR et al., 2007; LABER, 2006; BEISLAND et al., 2006; LABER, 2006; LIPWORTH; TARONE; MCLAUGHLIN, 2006).

Diversos eventos genéticos estão associados ao início da tumorigênese do ccRCC, dentre eles a perda da heterozigosidade no cromossomo 3p, inativação do gene *tríade frágil de histidina* (*FHIT*) e principalmente do gene supressor de tumor *Von Hippel-Lindau* (*VHL*) (LI et al., 2011). O gene *VHL*, quando mutado resulta na desregulação da hipoxia incluindo um aumento na produção do fator de crescimento endotelial vascular (VEGF), que por sua vez promove angiogênese, o crescimento tumoral e o desenvolvimento metástático (FINLEY; PANTUCK; BELLDEGRUN, 2011; PERIER et al., 2011). Além das alterações genéticas, mudanças epigenéticas também podem ocorrer no carcinoma renal de células (ARAI et al., 2012; PAIVA et al., 2011).

Parte do processo de formação do tumor pode ser atribuído a transição epitélio mesênquima (TSUJI; IBARAGI; HU, 2009). A Transição Epitélio Mesênquima (EMT) é definida como um programa chave do desenvolvimento, caracterizado pela redução da adesão célula-célula, das zônulas de oclusão, da polaridade apical-basolateral, e marcadores epiteliais, bem como a aquisição de mobilidade, células com formato fusiforme, e marcadores mesenquimais (JANG; BAEK; KIM, 2011; LIU, 2010; TSUJI; IBARAGI; HU, 2009). A transição epitélio mesênquima foi primeiramente reconhecida no processo de diferenciação no inicio da morfogênese no embrião (SHOOK; KELLER, 2003). O processo de EMT é um evento de múltiplos estágios, envolvendo um grande número de diferentes alterações genéticas e epigenéticas que resultam em um alto grau de mudanças celulares, convertendo células epiteliais completamente diferenciadas em células mesenquimais pouco diferenciadas, migratórias e invasivas (TIWARI et al., 2012).

A EMT está associada aos mecanismos de regeneração de tecidos adultos, cicatrização após uma lesão e invasão e metástase de tumores de origem epitelial. A EMT apresenta três classificações: a EMT tipo 1 refere-se a transformações que ocorrem nas configurações da biologia do desenvolvimento e EMT tipo 2 está ligada a reparação de tecidos adultos e fibroses e, por fim a tipo 3 se refere a difusão do câncer (HERTIG; FLIER; KALLURI, 2010).

A EMT tipo 1 controla a modificação celular de diversos estágios do desenvolvimento embrionário, incluindo a gastrulação, formação da crista neural e a

dissociação dos somitos e fusão dos tecidos craniofaciais (WALSH; NAWSHAD; MEDICI, 2011; KIM et al., 2011). O papel da EMT tipo 2 na regeneração de tecidos decorre da constatação de que proteínas específicas de fibroblastos são expressos pelas células epiteliais tubulares sob condição de estresse (HERTIG; FLIER; KALLURI, 2010).

As células epiteliais neoplásicas sofrem a transição epitélio mesênquima tipo 3, ou seja, perdem sua polaridade e tornam-se células mesenquimais móveis, o que auxilia no processo de metástase. Os estudos mostram que células de câncer submetidas a EMT passam a exibir propriedades de células tronco, perdem a senescência prematura e apoptose, o que contribui para a imunossupressão, revelando o papel da EMT na recorrência tumoral (KE et al., 2011; WU et al., 2011; BAO et al., 2011; GUTTILLA et al., 2011). Diversos trabalhos sugerem marcadores de EMT em carcinoma de células renais, como por exemplo, TGF-β, Claudin-1, HDAC2 e miR-34 (CRAENE; BERX, 2013; CATTO et al., 2011; NOH et al., 2009; FRITZSCHE et al., 2008; LIU et al., 2004).

Alguns estudos já têm estabelecido relação entre EMT e tumores de pâncreas, colorretal, mama, pulmão, próstata e carcinoma renal de células claras (REDOVA; SVOBODA; SLABY, 2011; WU et al., 2011; JANG; BAEK; KIM, 2011; BAO et al., 2011; TUN et al., 2010). Durante o processo de EMT diversas vias moleculares regulatórias podem agir, incluindo TGF-β, Wnt/β-catenina, receptores tirosina kinase e fator nuclear κB (JANG; BAEK; KIM, 2011; WU et al., 2011). Muitos fatores de transcrição tais como, Snail, Slug, Twist e Zeb podem causar mudanças transcricionais associadas a EMT em células tumorais (ISHIGAKI, et al., 2011). Tun e colaboradores (2010), por meio da técnica de *microarray*, tentaram elucidar os mecanismos moleculares envolvidos na transição epitélio mesênquima no carcinoma renal, identificando diversos genes com expressão aumentada em carcinoma renal de células claras (*N-caderina*, *fibronectina*, *fator transformador de crescimento-β1* e *vimentina*) quando comparado ao córtex renal normal.

As alterações epigenéticas podem influenciar se uma célula sofre ou não EMT e metástase (BAYLIN, 2005). Os eventos epigenéticos são mudanças hereditárias no padrão de expressão gênica associada a modificações no DNA ou proteínas da cromatina que não são acompanhadas por alterações na sequência do DNA (EGGER et al., 2004; HERMAN; BAYLIN, 2003). Essas modificações incluem a acetilação,

metilação, fosforilação, ubiquinação, sumoilação, carbonilação e a regulação por microRNAs (TANG et al., 2015). As alterações epigenéticas ocorrem em alta frequência no genoma, são reversíveis após tratamento com agentes farmacológicos e surgem em regiões definidas dentro de um gene (BAYLIN; OHM, 2006; FEINBERG; TYCKO, 2004). Essas características tornam a epigenética um campo muito atraente para a detecção do câncer e, diversos estudos têm mostrado múltiplos genes com diferentes tipos dessas alterações no tecido tumoral (SEPULVEDA et al., 2016; TAYLOR et al., 2016; KHAKPOUR et al., 2015; LI; MANSMANN, 2015; BENARD et al., 2014).

Os mecanismos epigenéticos clássicos, como a metilação do DNA e a modificação de histonas, regulam a expressão atuando na transcrição de genes e os microRNAs atuam principalmente como um mecanismo pós-transcricional (SAETROM; SNOVE; ROSSI, 2007).

A metilação do DNA é a adição de um grupo metil por um doador universal S-adenosilmetionina (SAM), para o átomo de carbono 5’ da citosina, presente no dinucleotídeo CpG (citosina – fosfato – guanina), resultando na formação de uma 5-metilcitosina (5meC). Esta reação é catalisada por uma enzima denominada DNA metiltransferase que inclui DNMT1, DNMT3A, DNMT3B e DNMT3L (AUWERA et al. 2009; SULEWSKA et al., 2007). Existem no genoma sequências curtas de DNA ricas em dinucleotídeos CpGs, conhecidas como ilhas CpG, frequentemente associadas ao sítio de início da transcrição dos genes e também pode ser encontradas na região intragênica e entre diferentes genes. Mais da metade dos genes do genoma de vertebrados apresentam essas regiões curtas ricas em CpGs (JONES, 2012; JONES; BAYLIN, 2007; COTRELL, 2004). Essas ilhas são encontradas, em sua maioria, na região 5’ do gene e normalmente mantêm-se não metiladas, desempenhando um importante papel no controle da expressão gênica (RAUCH; PFEIFER, 2005; USHIJIMA, 2005). A hipermetilação da região promotora é um evento relativamente comum em diversos tipos de tumores como, câncer de tireoide e carcinoma oral de células escamosas (WANG et al., 2013; RODRÍGUEZ-RODERO et al., 2013; HUANG et al., 2013).

A estrutura da cromatina também contribui na determinação da transcrição gênica. As DNMTs podem regular o silenciamento gênico por meio da metilação do

DNA e recrutando desacetilases de histonas (HDACs) e outras proteínas de ligação à cromatina promovendo a modificação de histonas (BAYLIN, 2005).

As modificações em proteínas histonas são consideradas mudanças dinâmicas com ocorrência de diferentes alterações dependendo do contexto celular (DAWSON; KOUZARIDES, 2012). A metilação e acetilação ocorrem na cauda N-terminal da histona e funciona de duas maneiras: primeiro por meio de alteração direta na estrutura da cromatina e segundo como um marcador molecular no recrutamento de complexos de modificação da cromatina (EZPONDA; LICHT, 2014; MOSASHVILLI et al., 2010). O estado de modificação de histonas influencia a conformação da cromatina e afeta a acessibilidade dos fatores de transcrição e outras proteínas o DNA, modificando assim a expressão do gene (VERVERIS et al., 2013). As alterações nas histonas como H3K27me3 e H3K4me3 são importantes no processo de desenvolvimento, e genes com essas modificações já foram revelados como contribuintes para a tumorigênese (DAWSON; KOUZARIDES, 2012). Diversos estudos já mostraram a importância da modificação de histonas em carcinoma de células renais (ROGENHOFER et al., 2012; ELLINGER et al., 2010; SELIGSON et al. 2009). Seligson e colaboradores (2009) revelaram que alterações do tipo H3K4me2, H3K18Ac e H3K9me2 estão correlacionadas com um pior prognóstico e com uma baixa probabilidade de sobrevivência em carcinoma de células renais. Um trabalho em larga escala com amostras de ccRCC revelou os níveis de metilação de H3K4 estão inversamente relacionados com o estágio avançado, grau e metástase nesta doença (ELLINGER et al., 2010).

Os microRNAs são moléculas de RNA com 19-24 nucleotídeos que se ligam em sequências complementares na região 3’ não traduzidas de seu RNA mensageiro alvo induzindo-o a degradação. Os transcritos de microRNAs são derivados de regiões intergênicas do genoma e foram conservados durante a evolução em muitos organismos, dentre eles, leveduras, moscas, plantas e seres humanos (LIU; MAO; ZHU, 2007; MALLORY et al, 2004).

A biogênese dos microRNAs é composta de múltiplos processos. Inicialmente, no núcleo celular ocorre a transcrição de um microRNA primário (Pri-miR), composto de dois segmentos de regiões de RNA pareados formando alças circulares. O Pri-miR é clivado por um complexo que inclui a enzima Drosha e uma proteína de ligação de RNA gerando um pequeno microRNA precursor (Pre-miR) (GREGORY et al., 2004;

LEE et al., 2003). O Pre-miR é então exportado para o citoplasma por meio da exportina-5, onde ocorre sua clivagem, pela ação da enzima Dicer, e consequente transformação em sua forma madura ainda composta por um RNA dupla fita (DU; ZAMORE, 2005). Este duplex de RNA é incorporado ao complexo de silenciamento induzido por RNA (RISC), no qual as duas fitas do microRNA são separadas. Uma das fitas permanece associada ao RISC e constitui o miRNA maduro funcional, ao passo que a fita complementar sofre degradação (FILIPOWICZ, 2005). O microRNA maduro induz o complexo RISC à ligação na região 3' não traduzida (3' UTR) do RNA mensageiro alvo, resultando em silenciamento gênico pós-transcricional pela degradação do mRNA ou por inibição da tradução (ZHANG; DAHLBERG; TAM, 2007).

Esses RNAs formam uma complexa rede de regulação gênica, pois um único microRNA pode ligar-se e regular diferentes alvos ou, diferentes microRNAs podem ligar-se de forma cooperativa para controlar um único mRNA alvo (KIM, 2005). Os microRNAs estão envolvidos em diversas vias regulatórias incluindo o controle de desenvolvimento, diferenciação e proliferação celular e apoptose (CALIN; CROCE, 2006).

Alterações na expressão de microRNAs vêm sendo relacionadas a diversos tipos de tumores, podendo funcionar como oncogenes ou gene supressores tumorais. Tais alterações já foram observadas em leucemia, glioblastoma, câncer de pulmão, mama, fígado, tireóide e pâncreas e câncer colorretal (ZHI et al.,2015; WANG et al., 2015; ZHANG et al., 2015; SIM et al., 2015; WANG et al., 2015; ZHOU et al., 2015; ZHAO; WANG, 2015)..

Os microRNAs podem atuar diretamente na EMT, como é o caso do miR-203 e miR-200 regulando SNAI1 e ZEB, respectivamente, contribuindo para a plasticidade das células epiteliais durante a diferenciação e a progressão do câncer (MOES et al., 2012). Em câncer de próstata o miR-29b pode suprimir o desenvolvimento de metástases pela regulação de sinalizações da EMT (RU et al., 2012). Em células de hepatoma, o miR-148a regula negativamente o alvo SNAI1, evitando a EMT e metástases (ZHANG et al., 2014).

Na tentativa de entender a biologia do carcinoma renal e identificar genes com expressão diferencial associados à EMT, neste trabalho analisamos diversos genes e selecionamos dois, *OCLN* e *GAS1*, e buscamos o seu mecanismo de regulação por meio

da atuação dos microRNAs, miR-122 e miR-34a, respectivamente. Além disso, verificou-se também a expressão diferencial do gene e da proteína SLC2A1.

O gene *OCLN*, responsável pela produção da proteína ocludina, é conhecido por estar presente em todas as junções *tight* (TJ). O gene *OCLN* está altamente expresso em TJ de células epiteliais de cólon normal, já em adenocarcinoma de cólon está com baixa expressão. (KARAGIANNIS et. al., 2013). A TJ é uma estrutura importante que determina a polaridade de células epiteliais e durante o processo de EMT ocorre a dissolução dessas junções (HARTEN et al., 2009). Em conjunto com junções aderentes e desmossomos, as TJs constituem um complexo juncional epitelial, estando localizados na parte apical da célula (IKENOUCHI et al., 2003). Nas TJs estão presentes as proteínas transmembranas ocludina e as claudinas, que ligadas ao citoesqueleto por meio de uma rede de proteínas, compõem as zônulas de oclusão 1 (THIERY; SLEEMAN, 2006; HOLLANDE et. al., 2002).

A ocludina está estruturalmente ligada ao receptor TGFβ, que também está localizado nas TJs. O fator TGFβ pode induzir sinalização patológicas nas células epiteliais diferenciadas e levar à mudanças fundamentais no fenótipo celular via EMT. Este processo pode estar no centro das principais causa de doenças potencialmente fatais do rim (PIERUCCI-ALVES et. al., 2011). Além disso, a ausência da proteína ocludina é característica de EMT em tumores derivados de células epiteliais simples (ISERI et al., 2011; AIJAZ; BALDA; MATTER, 2006). A baixa expressão do gene *OCLN* pode resultar em uma diminuição da adesão celular, redução da susceptibilidade à apoptose e alteração da diferenciação epidermal em carcinoma de células escamosas (RACHOW et al., 2013).

O miR-122 apresenta aproximadamente 22 nucleotídeos e regula a expressão de um grande número de mRNA alvo envolvidos nas mais diversas funções, como diferenciação celular e indução de apoptose (HSU; GHOSHAL, 2013; SZABO et al., 2013; JOPLING, 2012; HSU et al., 2012; TSAI et al., 2012) e tem como um dos alvos o gene *OCLN* (SENDI et al., 2015; YE et al., 2011). O papel do miR-122, envolvido na regulação do metabolismo lipídico, pode estar quando circulante em altos níveis relacionado com metástase em câncer de mama (WU et al., 2012; GIRARD et al., 2008). Além disso, no tecido hepático o miR-122 pode provocar um aumento e deslocamento nuclear, o que contribui para a interação entre o miR-122 e os membros da família Bcl, atuando com um regulador de apoptose endógena (JIN et a., 2014). Em

linhagem celular de adenocarcinoma colorretal, pode-se observar que a regulação do gene *OCLN* é realizada pelo miR-122, o que auxilia no aumento da permeabilidade do intestino (YE et al., 2011). Em 15 casos diagnosticados com carcinoma de células renais com característica de células claras e papilar o miR-122 foi encontrado com alta expressão (MUNARI et al., 2014). White e colaboradores (2011) e Zhou e colaboradores (2010) revelaram a alta expressão do miR-122 em 80 amostras de ccRCC. Foi demonstrado também que em câncer de mama a transferência de miR-122 para as células normais no nicho pré-metastático pode contribuir para reprogramação sistêmica do metabolismo de energia, facilitando a progressão da doença (FONG et al., 2015).

O gene de parada específica de crescimento 1 (*GAS1*) codifica uma proteína que se associa à porção externa membrana plamática celular por um fosfatidilinositol glicosídeo. Sabe-se que *GAS1* pode levar a parada do crescimento de células tumorais e induzir a apoptose, desse modo, a investigação do seu comportamento no tecido tumoral se faz necessário (ZARCO et al., 2012). O gene *GAS1* pode potencializar os sinais da via de sinalização Hedgehog, induzindo diversos fatores, como Notch e TGFβ, contribuindo indiretamente para a EMT (KATOH; KATOH, 2008). Jiang e colaboradores (2011) detectaram sua baixa expressão em pacientes com carcinoma colorretal que apresentaram recorrência tumoral.

A expressão de *GAS1* inibe a síntese de DNA e reduz a proliferação em linhagens de câncer de bexiga, pulmão e fibrosarcoma. Além disso, *GAS1* induz a apoptose em células de gliomas e neuroblastoma por meio da ativação da caspase-3 e 9 (ZARCO et al., 2012; DOMINGUEZ-MONZON et al., 2009; STEBEL et al., 2000; EVDOKIOU; COWLED, 1998). Pacientes com câncer gástrico e com baixa expressão do gene *GAS1* apresentam menor tempo de sobrevida quando comparados a outros pacientes com expressão normal desse gene (WANG et al., 2012).

O miR-34a, membro da família dos miR-34, é um alvo transcricional do gene p53, atuando em diversos tipos de cânceres e apresentando um papel fundamental em processos biológicos como diferenciação celular, proliferação e sobrevivência (LEWIS; BURGE; BARTEL, 2005). O miR-34a é capaz de regular negativamente a proteína Bcl-2 em células de câncer de pulmão e induzir apoptose pela ativação da proteína apoptótica caspase-3 em células de câncer de pâncreas (BANDI; VASSELLA, 2011; NALLS et al., 2011). A alta expressão do miR-34a já foi associada a 15 casos

diagnosticados com carcinoma renal (MUNARI et al., 2014). O miR-34a em carcinoma de células renais apresenta um papel de supressor tumoral por meio da regulação do gene YY1 (WENG et al., 2013). Um trabalho realizado por Ma, Qin e Cui (2013) demonstrou que a regulação do gene *GAS1* em carcinoma papilar de tireoide ocorre por meio do miR-34a, levando à proliferação celular e suprimindo da apoptose. A regulação de *GAS1* pelo miR-34a contribui também para proliferação mesangial e hipertrofia glomerular em diabete nefropática (ZHANG et al., 2014). Em gliomas a alta expressão do miR-34a contribui para a malignidade do tumor (RATHOD et al., 2014).

O perfil de expressão dos microRNAs pode fornecer informações para prognóstico clínico e uma terapia mais efetiva (GARZON; MARCUCCI, 2012; CHO, 2010; ZENG, 2006).

O gene *SLC2A1* codifica uma proteína denominada transportador de glicose tipo 1, fundamental para as vias de metabolismo celular energético. Esta proteína é capaz de desenvolver um fluxo bidirecional de glicose de acordo com o gradiente do substrato. SLC2A1 está com alta expressão em diferentes tipos de carcinomas, incluído hepático, pulmão, endométrio, oral e câncer de mama (KRZESLAK et al., 2012; AMANN et al., 2009; WACHI; YONEDA; WU, 2005; NOGUCHI et al., 2000; KAWAMURA et al., 2001). Estas observações sugerem que este gene pode contribuir para o desenvolvimento de diversos tumores (YAN et al., 2015).

Foi mostrado que osteossarcoma e suas metástases apresentam a hiperexpressão do gene *SLC2A1*, o que contribui para o processo de proliferação das células tumorais (CIFUENTES, et al., 2011). A proteína SLC2A1 está dentro de uma variedade de marcadores para a detecção dos diferentes tipos de câncer de mama, sendo que sua expressão detectada por imuno-histoquímica, é mais frequente em carcinoma ductal *in situ* do que em câncer de mama invasivo (VERMEULEN et al., 2013; VERMEULEN et al., 2012).

O progresso observado na análise de amostra de tumores por análise da expressão gênica e seus mecanismos de regulação tem mostrado grande eficiência no fornecimento de novas informações sobre a ativação de vias de sinalização de vários processos, tais como evasão de apoptose, angiogênese e potencial metastático.

OBJETIVOS

OBJETIVOS

Tendo em vista o exposto, o objetivo geral deste projeto foi analisar a expressão de genes e proteínas relacionados ao processo de EMT, além de analisar o possível mecanismo de regulação de genes com baixa expressão em carcinoma renal de células claras e linhagem celular de carcinoma renal. Para isso, foram utilizadas metodologias moleculares, procurando atender os seguintes objetivos específicos:

1. Selecionar genes envolvidos na transição epitélio mesenquima, diferenciação e proliferação celular em amostras de carcinoma renal de células claras e em linhagem celular de carcinoma renal;

2. Avaliar a expressão proteica dos genes hiperexpressos em carcinoma renal de células claras e em linhagem celular de carcinoma renal;

3. Analisar se o perfil de metilação do DNA, as modificações de histonas e os microRNAs estão regulando a expressão dos genes hipoexpressos em amostras de carcinoma renal de células claras e em linhagen celular de carcinoma renal.

ARTIGO CIENTÍFICO

ARTIGO CIENTÍFICO (SUBMETIDO À *JOURNAL OF CANCER*)

Epithelial-to-mesenchymal transition related genes are differentially expressed in clear cell renal cell carcinoma

André Luis Giacometti Conceição[1], Camila Tainah da Silva[2], Rodolfo Miglioli Badial[1], Marina Curado Valsechi[1], Bruna Stuqui[1], Jéssica Domingues Gonçalves[2], Miriam Galvonas Jasiulionis[2], Marilia de Freitas Calmon[1], Paula Rahal[1*].

1. Laboratory of Genomics Studies, UNESP, São José do Rio Preto, SP, Brazil.
2. Pharmacology Department, Federal University of São Paulo, UNIFESP, São Paulo, SP, Brazil.

* Corresponding author: Paula Rahal, PhD, Department of Biology, Instituto de Biociências, Letras e Ciências Exatas – IBILCE/ UNESP, Street: Cristóvão Colombo, 2265, Zip/Postal Code: 15054-000 – City: São José do Rio Preto, SP, Brazil. E-mail address: rahalp@yahoo.com.br, Phone: +55 17 3221-2379, Fax: +55 17 3221-2390.

Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of kidney cancer. This carcinoma is histologically characterized by the presence of clear and abundant cytoplasm. The initial process of ccRCC metastatic tumor formation can be attributed to epithelial-mesenchymal transition (EMT). In this study, we sought to identify genes differentially expressed in ccRCC and build a molecular profile of this cancer. We selected genes described in the literature to be related to EMT, cellular differentiation and proliferation. We analyzed the gene and protein expression by quantitative PCR and immunohistochemistry, respectively, and examined possible epigenetic mechanisms that regulate their expression in ccRCC samples and cell lines. *OCLN* and *GAS1* genes were under-expressed in ccRCC, and we report that miR-122 and miR-34a, respectively, may regulate their expression in this cancer. Furthermore, we showed by quantitative PCR (qPCR) and immunohistochemistry that *SLC2A1* was significantly over-expressed in ccRCC. The set of genes identified in this study furthers our understanding of the molecular basis and development of ccRCC.

Keywords: Clear cell renal cell carcinoma, gene expression, epigenetics

Introduction

Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of kidney cancer in adults (70 – 75% of cases) [1]. Approximately 30% of early stage patients develop metastases after surgery for localized disease [2]. This carcinoma is histologically characterized by the presence of clear and abundant cytoplasm [2, 3]. The aggressive characteristics of renal clear cell carcinoma interfere in the efficiency of conventional treatment approaches, such as chemotherapy and radiotherapy [4].

Part of the tumor formation process can be attributed to epithelial-mesenchymal transition (EMT) [5]. During EMT, epithelial cells lose their junctions (tight junctions, adherens junctions, desmosomes and gap junctions) and apical-basal polarity, undergo a change in the cell shape signaling programs, reprogram gene expression, increase cellular motility and develop an invasive phenotype [6, 7]. Recent work identified various EMT markers in renal carcinoma, such as TGF-β, Claudin-1, HDAC2 and miR-34 [8-11].

Mutations or deletions associated with tumor suppressor genes may cause the loss or inactivation of these negative regulators. However, the loss of function may be caused by epigenetic changes [12], including acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, carbonylation, and regulation of microRNAs [13]. Epigenetic alterations occur at a high frequency, are reversible upon treatment with pharmacological agents and arise at defined regions within a gene [14, 15]. These features make epigenetics a very attractive field for cancer detection and several studies have shown multiple genes with different types of epigenetic alterations in tumor tissue [16-18].

Analyzing gene expression and regulatory mechanisms in tumor samples has shown great efficiency in providing new information on signaling pathway activation of several processes, such as evasion of apoptosis, angiogenesis and metastatic potential.

In the present study, we selected genes described in the literature as having a relationship with epithelial-mesenchymal transition, cellular differentiation and proliferation. We analyzed their expression by quantitative PCR and immunohistochemistry, and examined the possible epigenetic mechanisms that regulate genes in ccRCC samples and cell line. *OCLN* and *GAS1* were under-expressed in ccRCC and miR-122, and miR-34a may regulate their expression in this cancer.

Furthermore, we showed by qPCR and immunohistochemistry that *SLC2A1* was significantly overexpressed in ccRCC samples. These genes may be involved in the molecular biology and development of the disease and analysis of them may contribute to insights for new prognoses, treatments and understanding this tumor type.

Material and Methods

Clear cell renal cell carcinoma samples

The samples were collected from 101 patients diagnosed with clear cell renal cell carcinoma, including 45 fresh samples of ccRCC, 56 paraffin-embedded samples of ccRCC, 2 fresh-frozen, histologically normal renal tissue samples and 24 paraffin-embedded samples of histologically normal renal tissues, all of which were confirmed by pathologists. The samples were obtained from the Hospital de Base at Faculdade de Medicina de São José do Rio Preto, Sao Paulo, Brazil. The use of patient-derived material was approved by the Research Ethics Committee of the IBILCE-UNESP and FAMERP, Sao Paulo, Brazil, and written consent was obtained from all patients. Tissues were obtained from patients undergoing tumor resection surgery, and a diagnosis of clear cell renal cell carcinoma was verified post-operatively using histopathology. The samples were classified according to the criteria provided by the International Union against Cancer [19].

Cell lines and Treatments

The 786-O (primary clear cell adenocarcinoma) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and HaCaT (an immortal human keratinocyte cell line) was kindly provided by Luisa Lina Villa (Department of Radiology, Center on Translational Oncology Investigation, São Paulo State Cancer Institute, Universidade de São Paulo, Brazil). 786-O cells were cultured in RPMI1640 medium (Gibco by Life Technologies, Grand Island, NY, USA), and HaCaT cells were cultured in DMEM medium (Gibco by Life Technologies, Grand Island, NY, USA). The media were supplemented with 10% fetal bovine serum (Cultilab, SP, Brazil), 100 U/mL of penicillin (Invitrogen, Grand Island, NY, USA) and 100 µg/mL of streptomycin (Invitrogen, Grand Island, NY, USA). Cell cultures were grown in a 37°C and 5% CO_2 atmosphere. 786-O cells were plated in 6-well plates, cultured for 24

hours, and treated for 72 h with fresh 5 μM, 2 μM or 1 μM of 5-Aza-dC (Sigma, St Louis, MO, USA) dissolved in phosphate buffered saline. Due to its chemical instability, fresh medium with 5-Aza-dC was added every 24 h. For the Trichostatin A (TSA) experiments, the cells were seeded in 6-well plates, cultured for 24 hours, and treated for 24 hours with fresh 1,000 nM, 500 nM, 300 nM, 200 nM or 100 nM of TSA (Sigma, St Louis, MO, USA) dissolved in DMSO. To assess the combined effect of both drugs, we performed the co-treatment of cells with 5 μM of 5-Aza-dC and 1,000 nM of TSA using the following protocol: 5-Aza-dC was added for 72 h, after which it was removed and TSA was added for an additional 24 h. All experiments were performed in triplicate.

Cytotoxicity assay

786-O cells were seeded into 96-well plates at a density of 10^3 cells for 5-Aza-dC treatment, 10^4 cells for TSA and 5 x 10^3 cells for co-treatment. After the 5-Aza-dC and TSA treatments, 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Aldrich, St Louis, MO, USA) was added to each well and incubated for 30 min at 37ºC. Then, the MTT was removed and 100 μL of 100% DMSO (Sigma Aldrich, St Louis, MO, USA) was added to each well and the absorbance was measured at 562 nm. Each experiment was performed in triplicate and in two independent assays.

RNA and DNA Extraction

Genomic DNA and total RNA extraction were performed with TRIzol® Reagent (Life Technologies, Grand Island, NY, USA) using both cell lines, the 45 fresh samples of ccRCC and 2 fresh-frozen, histologically normal renal tissue samples, following the manufacturer's instructions. The DNA and RNA quality was verified by electrophoresis through an agarose gel and stained with ethidium bromide. For quantitative real-time PCR, 5 μg of total RNA from each sample were used to synthesize cDNA with a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The cDNA quality was checked by PCR of the housekeeping gene *β-ACTIN*. The PCR products were analyzed by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

Selection of Genes

We analyzed the differential expression of the following genes by qPCR: *E-Cadherin (CDH1), N-Cadherin (CDH2), Claudin 1 (CLDN1), Cathepsin B (CTSB), Platelet-activating factor acetylhydrolase 2 (PAFAH2), Growth arrest-specific 1 (GAS1), Hypoxia inducible factor 1α (HIF1α), Hypoxia inducible factor 2α (HIF2α), Laminin β1 (LAMβ1), Occludin (OCLN), Phosphoglycerate dehydrogenase (PHGDH), Solute carrier family 2 member 1 (SLC2A1), Transforming growth factor β1 (TGFβ1), Tight junction protein 1 (TJP1)* and *Vimentin (VIM)*. The genes were selected according to their role in the EMT process, cellular differentiation and proliferation. The primers sequences are provided in Table S1.

Quantitative Real-time Polymerase Chain Reaction

The qPCR amplification was performed with Power SYBR Green and an ABI 7300 Real-time PCR System (Applied Biosystems, Warrington, UK). In brief, the reaction mixture (20 μL of total volume) contained 25 ng of cDNA, gene-specific forward and reverse primers and 10 μL of 2x Quantitative SYBR Green PCR Master Mix. The samples were tested in triplicate. The relative expression of each specific gene was calculated using the following previously published [20] formula:
$R = (E\ target)^{\Delta Ct\ target\ (control\ -\ sample)} / (E\ endogenous)^{\Delta Ct\ endogenous\ (control\ -\ sample)}$. A 2-fold change was set as the cutoff. Gene expression was analyzed in forty-five ccRCC samples and in the 786-O cells after treatment and HaCaT. Two normal renal fresh-frozen tissue samples were used as the normal reference (control group). Non-treated cell lines were used as reference samples, and *GAPDH* was selected as the endogenous control gene.

Bisulfite Sequencing

Genomic DNA was subjected to a sodium bisulfite treatment to modify unmethylated cytosine to uracil, as described by Calmon et al. [21]. Bisulfite-treated DNA was amplified by PCR, using primers designed to amplify CpG-rich regions located at the 5' region of *OCLN* (-438 to +1232, relative to the transcription start site (TSS) and encompassing 164 CpG dinucleotides) and *GAS1* (-485 to +1518, relative to the TSS and encompassing 269 CpG dinucleotides). Primer sequences are provided in Table S1. The amplified products were cloned using a CloneJet PCR Cloning Kit

(Thermo Scientific, Carlsbad, CA, USA). Five positive clones from the 786-O cell line before and after treatment with 5-Aza-dC, one ccRCC sample and one normal renal sample were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing kit and an ABI3130XL sequencer, according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The methylation percentage for each sample was calculated as the proportion of unconverted CpG dinucleotides among all of the CpG dinucleotides analyzed from the five positive clones.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP-IT® High Sensitivity Kit, as described by the manufacturer (Active Motif, Carlsbad, CA, USA). The 786-O and HaCaT cells were cross-linked with 1% formaldehyde for 10 min at room temperature and nuclei were isolated. Isolated nuclei were sonicated on ice to break the chromatin into approximately 200–1200 bp fragments. Soluble chromatin was used in immunoprecipitation with H3K4me3, H3K27me3, H3K4me2, and H3K9ac antibodies (Thermo Scientific, Rockford, IL, USA). IgG was used as a negative control (abcam, Cambridge, MA, USA) and immune complexes were absorbed with protein G magnetic beads. After reversing the cross-linking and treating with proteinase K, the immunoprecipitated DNA was analyzed on a gene-specific basis using quantitative PCR (primers sequences in table 2). The quality control ChIP was performed using a ChIP-IT® qPCR Analysis Kit (Active Motif, Carlsbad, CA, USA) with Human Negative Control Primer Set 1 and Human Positive Control Primer Set GAPDH-2, as described by the manufacturer.

MicroRNA Analysis

Two microRNA databases, miRBase (www.mirbase.org) and TargetScanHuman (www.targetscan.org), were used to select microRNAs that target the *OCLN* and *GAS1* mRNA. For microRNA expression analysis, total RNA was reverse transcribed into cDNA using miR-34a, miR-122 and U48 specific steam-loop primers (the primer sequences are listed in Table S2). Expression of mature miR-34a and miR-122 was quantified as described in the qPCR methods. The microRNA expression was analyzed in 30 clear cell renal cell carcinoma samples and in the cell lines 786-O and HaCaT.

Two normal renal fresh-frozen tissue samples were used as a normal reference. Each sample was run in triplicate, and the miRNA expression levels were normalized to U48.

Tissue array construction and immunohistochemistry

Tissue microarrays were constructed using a tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Briefly, the slides were reviewed by a pathologist, and areas containing each category were annotated on the H&E slides. Fifty-six 1.0 mm in diameter cylindrical tissue cores from the ccRCC tumor samples and twenty-four from the tumor margins were taken from the corresponding regions of the paraffin blocks and transplanted into a recipient paraffin block.

For immunohistochemical staining, all of the paraffin-embedded sections were cut to 4 μm in thickness and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. Antigen retrieval was performed using sodium citrate buffer (pH 6,0) for 20 minutes at 96ºC in a steam pan. The sections were then incubated with SLC2A1 polyclonal primary antibody (1:200) (Abcam, Cambridge, MA, USA) diluted in 1% BSA at 4ºC overnight. After incubation with primary antibody, the sections were incubated with a biotinylated secondary antibody (Santa Cruz Biotechnology, Paso Robles, CA, USA) and then exposed to streptavidin complex (HRP ready-to-use, DakoCytomation, Carpinteria, CA, USA). Positive staining was identified using DAB substrate color detection (Dako, Cambridge, UK). The sections were counterstained with Hematoxylin.

The SLC2A1 densitometric analyses were conducted with an Axioskop II microscope (Zeiss, Oberkochen, Germany) using the Axiovision^{TM} software (Zeiss). For these analyses, 20 different points were analyzed from three different fields of each tumor fragment and the average immunoreactivity intensity (arbitrary units, AU) was recorded.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 Software (GraphPad Software Inc., San Diego, CA, USA). The relative expression levels detected by qPCR in ccRCC were transformed into natural logarithms. The Wilcoxon signed-rank test was applied to compare the gene expression levels in tumor tissues and normal

tissues. The Mann–Whitney U Test and Wilcoxon signed-ranks test were used to compare the protein expression levels detected by immunohistochemistry.

Results

Identification of differentially expressed genes in ccRCC

To identify genes differentially expressed in ccRCC samples and the 786-O cell line, we performed qPCR for the following target genes: *CDH1*, *CDH2*, *CLDN1*, *CTSB*, *PAFAH2*, *GAS1, HIF1α, HIF2α, LAMβ1, OCLN, PHGDH, SLC2A1, TGFβ1, TJP1* and *VIM*.

CDH2, *CTSB*, *GAS1*, *HIF1α*, *LAMβ1*, *OCLN*, *PHGDH*, *PAFAH2* and *TJP1* were significantly downregulated in the ccRCC samples when compared to the reference samples. *SLC2A1* was significantly overexpressed in tumor tissues when compared to normal renal tissues. *CDH1*, *TGFβ1*, *VIM*, *CLDN1* and *HIF2α* were not differentially expressed (Figure 1A).

CDH1, *CTSB*, *GAS1* and *OCLN* were significantly downregulated in 786-O cells. *SLC2A1* and *VIM* were significantly overexpressed in 786-O cells. *CDH2*, *CLDN1*, *HIF1α*, *HIF2α*, *LAMβ1*, *PAFAH2*, *PHGDH*, *TGFβ1* and *TJP1* were not differentially expressed (Figure 1B).

OCLN and *GAS1* were chosen for epigenetic analysis due to the presence of CpG islands in their promoter regions and the observed downregulation in both the ccRCC samples and 786-O cells. *SLC2A1* was selected for immunohistochemical analysis because it was overexpressed in both the ccRCC samples and 786-O cells.

DNA methylation and histone acetylation analysis

The methylation status of CpG islands at the 5' region of the *OCLN* and *GAS1* genes was investigated by bisulfite sequencing in ccRCC samples, normal renal tissue and 786-O cells before and after 5-Aza-dC treatment. The bisulfite sequencing was performed in the -438 to +1232 region of *OCLN* and the -485 to +1518 region of *GAS1*.

The methylation levels of *OCLN* and *GAS1* were not significantly reduction in 786-O cells before and after 5 μM of 5-Aza-dC treatment (24.39% to 22.19% for *OCLN* and 22.5% to 22.23% for *GAS1*). The methylation level of the genes compared between

the ccRCC samples and normal renal samples did not differ significantly, 4% to 1% for *OCLN* and 1.8% to 0% for *GAS1* (Figure 2).

An MTT assay was performed to detect possible effects of 5-Aza-dC and TSA treatment on 786-O cell viability. All of the drug concentrations tested showed viability greater than 75%, including co-treatment (Figure 3).

The cell growth was inhibited in a dose-dependent manner by 5-Aza-dC treatment in 786-O cells. The expression of *OCLN* mRNA was higher in the cells treated with 5 μM and 2 μM of 5-Aza-dC than in the untreated cells (Figure 4A). There was no difference in the *GAS1* mRNA expression after treatment with any 5-Aza-dC concentration (Figure 4A).

Similar to the 5-Aza-dC treatment, TSA treatment showed a better response at the highest concentration (1,000 nM). The *OCLN* gene was upregulated in the 786-O cells after 1,000 nM TSA treatment when compared to untreated cells (Figure 4B). Other TSA concentrations were not able to significantly upregulate *OCLN* expression. The *GAS1* expression levels were unchanged after TSA treatment in 786-O cells when compared to untreated cells (Figure 4B).

The 5-Aza-dC (5 μM) and TSA (1000 nM) co-treatment efficiently upregulated *OCLN* expression in 786-O cells. However, *GAS1* gene expression was not altered after co-treatment (Figure 4C).

ChIP

Because we could not observe any difference in the promoter region methylation levels of the genes after 5-Aza-dC treatment, we investigated whether the loss of *OCLN* and *GAS1* expression in 786-O cells could be due to histone modifications. For this, we performed ChIP analyses for H3K4me3, H3K27me3, H3K4me2 and H3K9ac marks at the *OCLN* and *GAS1* promoters in the 786-O and HaCaT cell lines.

In both cell lines, ChIP followed by qPCR analysis showed that all of the histone modifications were reduced or absent in the promoter regions of *OCLN* and *GAS1*. It was not possible to differentiate the relative occupancy of the marks studied between the normal and tumor cell lines. The Human Negative Control Primer Set 1 and Human Positive Control Primer Set GAPDH-2 served as negative and positive controls, respectively (Figure 5).

microRNA analysis

We performed stem loop reverse transcription PCR (RT-qPCR) to quantify miR-122 and miR-34a expression in the 786-O and HaCaT cell lines. The relative expression of miR-122 and miR-34a in 786-O cells was higher than in HaCaT cells (Figure 6). Both microRNAs were significantly overexpressed in tumor tissues when compared to normal renal tissues. These results suggest that upregulation of miR-122 and miR-34a might be, at least in part, involved in the downregulation of *OCLN* and *GAS1* in ccRCC.

Immunohistochemistry

The SLC2A1 protein expression was investigated on ccRCC tissue array by immunohistochemistry.

The ccRCC tissues showed higher expression of SLC2A1 protein ($p<0.0001$) relative to normal tissues. Figure 7 shows the SLC2A1 immunoreactivity and the densitometric data.

Discussion

Clear cell renal cell carcinoma (ccRCC) accounts for the majority of renal carcinoma related deaths [2]. Some patients who develop metastases present resistance to systemic chemotherapy and radiation therapy, contributing to the development of therapies that target the molecular basis of the cancer [22, 23].

Several prognostic models are used to predict the survival of ccRCC patients, including clinicopathologic parameters and protein, genetic and epigenetic biomarkers [24-26]. In the present study, we investigated the epigenetic regulatory mechanisms of *OCLN* and *GAS1*, genes that may contribute to epithelial-mesenchymal transition, cellular differentiation and proliferation in renal cell carcinoma.

The *OCLN* gene is responsible for producing the occludin protein, which is present in all of the tight junctions. Occludin has been implicated in tight junction permeability and, in particular, to the regulation of size-selective diffusion [27-29]. The transmembrane proteins occludin and claudin are present in the tight junctions, which are connected to the cytoskeleton via a network of proteins, such as zonula occludens-1 [30]. This epithelial junctional complex, together with adherens junctions and desmosomes, is located at the most apical part of the complex [31]. The tight junctions

determine epithelial cell polarity and undergo dissolution during cancer progression that is accompanied by cytoskeletal changes [6].

Downregulation of *OCLN* was observed in liver tumors, breast cancer, endometrial and lung carcinomas [32-35]. A study of human cutaneous squamous cell carcinoma observed the loss of OCLN and related this loss to cell-cell adhesion, apoptosis and proliferation [36]. *OCLN* was found to be partially methylated in the promoter and endogenous region of a breast cancer cell line, and treatment with 5-Aza-dC and TSA could induce re-expression of this gene [37]. In our study, we observed hypermethylation in approximately 20% of the endogenous region, although, treatment with 5-Aza-dC did not change the global methylation profile of the *OCLN* gene in the ccRCC cell line. However, we demonstrated that the effect of 5-Aza-dC treatment and synergistic effect of 5-Aza-dC and TSA treatment in 786-O cells was effective in reactivating *OCLN* gene expression. Thus, 786-O cells showed a methylation profile in the *OCLN* promoter, despite the suggestion of alternate modes of reactivation base upon *OCLN* expression. To further investigate whether increased *OCLN* expression in 786-O cells could be due to chromatin modifications, we performed ChIP for H3K27me3, H3K4me3, H3K4me2 and H3K9ac marks in the *OCLN* promoter in 786-O cells. ChIP analysis did not show any differences between H3K27me3 silencing marks or changes in H3K4me3, H3K4me2 and H3K9ac activation marks in the *OCLN* gene promoter region. H3K4me1, H3K4me2 and H3K4me3 marks inform potential prognosis because their increase was inversely correlated with lymph node involvement and distant metastasis in patients with renal cell carcinoma [38].

Another potential regulatory mechanism that may influence *OCLN* expression is microRNAs. miR-122 has been reported to regulate *OCLN* expression in intestinal tissue, which contributes to increased intestinal permeability [39]. In this study, miR-122 expression levels were higher in the ccRCC cell line and tumor samples, suggesting that the increase in microRNA expression may contribute to the downregulation of the *OCLN* gene. miR-122 expression was upregulated in 15 cases of clear cell papillary renal cell carcinoma and in 80 samples of ccRCC [40-42]. In breast cancer, high miR-122 levels have been associated with metastasis, likely by increasing nutrient availability in the pre-metastatic niche [43].

GAS1 is a membrane-associated protein; it is tethered to the membrane through a glycosyl-phosphatidylinositol anchor [44, 45]. GAS1 contributes to inhibitory signal

transduction and interferes with cell proliferation due to the inhibition of DNA synthesis during G0 to S [44, 46]. The Hedgehog pathway is an essential regulator of carcinogenesis, and GAS1 protein participates in the Hedgehog signaling pathway through binding to Hedgehog ligands and enhancing the signaling, thus indirectly inducing EMT [47-49].

Reduced *GAS1* expression was positively associated with the survival time in gastric cancer cells [50]. The downregulation of the *GAS1* gene has been described in colorectal cancer, melanoma and prostate cancer metastasis [51-53].

In this present study, the global methylation profile of *GAS1* in ccRCC cells was unchanged before and after 5-Aza-dC treatment. Similarly, there were no gene expression modifications after treatment with TSA or co-treatment with TSA and 5-Aza-dC. Sacilotto et al. (2011) [54] suggested that *GAS1* gene transcription is modulated by chromatin remodeling and histone modifying complex recruitment to the *GAS1* promoter in hepatic cells. However, our chromatin immunoprecipitation assays did not show a difference in the H3K27me3 silencing mark, nor were there differences in the H3K4me3, H3K4me2 and H3K9ac activation marks in the *GAS1* gene promoter region in ccRCC cells. It is possible that other regulatory mechanisms are altering the expression of *GAS1* in ccRCC, such as microRNAs.

GAS1 gene regulation in papillary thyroid carcinoma occurs through miR-34a, leading to cell proliferation and apoptotic suppression [55]. miR-34a regulates mesangial proliferation and glomerular hypertrophy by directly inhibiting *GAS1* in early diabetic nephropathies [56]. In this study, we measured miR-34a expression levels, which target the *GAS1* gene, in ccRCC cell lines and tumor samples and suggest that the increase in this microRNA may contribute to the downregulation of *GAS1*. miR-34a was upregulated in eight ccRCC samples and renal carcinoma cell lines, establishing an association between this miR and multiple targets [57, 58]. miR-34a overexpression supports cell proliferation in the majority of cancers, suggesting an unexpected link between cancer and neuronal and endocrine cell metabolism [59].

The *SLC2A1* gene is over-expressed in different types of cancer, including lung, liver and breast cancers [60-62]. SLC2A1 facilitates glucose transport and is present in the epithelium and endothelium, contributing to proliferation [60, 63, 64]. In this study, *SLC2A1* was upregulated by both qPCR and immunohistochemistry in ccRCC samples and 786-O cells. Cifuentes et al. [65] identified that SLC2A1 and glucose transport are

regulated by insulin and therefore may be an important target for conventional pharmaceutical therapy and gene therapy in the treatment of osteosarcoma. The SLC2A1 protein belongs to a class of markers that can detect breast cancer by molecular imaging with more common immunoreactivity in ductal carcinoma in situ than in invasive breast cancer [66, 67].

In summary, the silencing of *OCLN* and *GAS1* may contribute to tumor progression because of a possible relationship between these genes and cell-cell adhesion, apoptosis, proliferation and EMT. We suggest that the increase in miR-122 and miR-34a expression may contribute to the downregulation of the *OCLN* and *GAS1* genes, respectively. Overexpression of the *SLC2A1* gene was higher in ccRCC than in the non-neoplastic samples. The expression of these genes may be involved in EMT in addition to other cellular processes that contribute to tumor development. Once these genes have been characterized in ccRCC, molecular genetic tools may be used to explore the biological process involved, thus improving diagnosis, treatment, and patient outcomes. Further studies are required to evaluate whether the identified genes are specifically altered in ccRCC or if they might be deregulated in other tumors.

Funding

The study was supported by São Paulo State Research Foundation – FAPESP (2012/08853-0) and National Council for Research - CNPq.

Competing interests

The authors disclose that they have no financial interests in the subject of paper.

References

1. Shuch B, Amin A, Armstrong AJ, Eble JN, Ficarra V, Lopez-Beltran A, et al. Understanding pathologic variants of renal cell carcinoma: distilling therapeutic opportunities from biologic complexity. European urology. 2015; 67: 85-97. doi:10.1016/j.eururo.2014.04.029.

2. Cohen HT, McGovern FJ. Renal-cell carcinoma. The New England journal of medicine. 2005; 353: 2477-90. doi:10.1056/NEJMra043172.

3. Kuroda N, Hosokawa T, Michal M, Hes O, Sima R, Ohe C, et al. Clear cell renal cell carcinoma with focal renal angiomyoadenomatous tumor-like area. Annals of diagnostic pathology. 2011; 15: 202-6. doi:10.1016/j.anndiagpath.2010.03.003.

4. Nagata M, Sakurai-Yageta M, Yamada D, Goto A, Ito A, Fukuhara H, et al. Aberrations of a cell adhesion molecule CADM4 in renal clear cell carcinoma. International journal of cancer Journal international du cancer. 2012; 130: 1329-37. doi:10.1002/ijc.26160.

5. Tsuji T, Ibaragi S, Hu GF. Epithelial-mesenchymal transition and cell cooperativity in metastasis. Cancer research. 2009; 69: 7135-9. doi:10.1158/0008-5472.CAN-09-1618.

6. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nature reviews Molecular cell biology. 2006; 7: 131-42. doi:10.1038/nrm1835.

7. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nature reviews Cancer. 2002; 2: 442-54. doi:10.1038/nrc822.

8. Liu Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. Journal of the American Society of Nephrology : JASN. 2004; 15: 1-12.

9. Fritzsche FR, Oelrich B, Johannsen M, Kristiansen I, Moch H, Jung K, et al. Claudin-1 protein expression is a prognostic marker of patient survival in renal cell carcinomas. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008; 14: 7035-42. doi:10.1158/1078-0432.CCR-08-0855.

10. Noh H, Oh EY, Seo JY, Yu MR, Kim YO, Ha H, et al. Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-beta1-induced renal injury. American journal of physiology Renal physiology. 2009; 297: F729-39. doi:10.1152/ajprenal.00086.2009.

11. Catto JW, Alcaraz A, Bjartell AS, De Vere White R, Evans CP, Fussel S, et al. MicroRNA in prostate, bladder, and kidney cancer: a systematic review. European urology. 2011; 59: 671-81. doi:10.1016/j.eururo.2011.01.044.

12. Baylin SB. DNA methylation and gene silencing in cancer. Nature clinical practice Oncology. 2005; 2 Suppl 1: S4-11. doi:10.1038/ncponc0354.

13. Tang J, Zhuang S. Epigenetics in acute kidney injury. Current opinion in nephrology and hypertension. 2015; 24: 351-8. doi:10.1097/MNH.0000000000000140.

14. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nature reviews Cancer. 2006; 6: 107-16. doi:10.1038/nrc1799.

15. Feinberg AP, Tycko B. The history of cancer epigenetics. Nature reviews Cancer. 2004; 4: 143-53. doi:10.1038/nrc1279.

16. Benard A, Goossens-Beumer IJ, van Hoesel AQ, de Graaf W, Horati H, Putter H, et al. Histone trimethylation at H3K4, H3K9 and H4K20 correlates with patient survival and tumor recurrence in early-stage colon cancer. BMC cancer. 2014; 14: 531. doi:10.1186/1471-2407-14-531.

17. Khakpour G, Pooladi A, Izadi P, Noruzinia M, Tavakkoly Bazzaz J. DNA methylation as a promising landscape: A simple blood test for breast cancer prediction. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2015; 36: 4905-12. doi:10.1007/s13277-015-3567-z.

18. Li J, Mansmann UR. A microRNA molecular modeling extension for prediction of colorectal cancer treatment. BMC cancer. 2015; 15: 472. doi:10.1186/s12885-015-1437-0.

19. Fisseler-Eckhoff A. [New TNM classification of malignant lung tumors 2009 from a pathology perspective]. Der Pathologe. 2009; 30 Suppl 2: 193-9. doi:10.1007/s00292-009-1195-3.

20. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research. 2001; 29: e45.

21. Calmon MF, Colombo J, Carvalho F, Souza FP, Filho JF, Fukuyama EE, et al. Methylation profile of genes CDKN2A (p14 and p16), DAPK1, CDH1, and ADAM23 in head and neck cancer. Cancer genetics and cytogenetics. 2007; 173: 31-7. doi:10.1016/j.cancergencyto.2006.09.008.

22. Audenet F, Yates DR, Cancel-Tassin G, Cussenot O, Roupret M. Genetic pathways involved in carcinogenesis of clear cell renal cell carcinoma: genomics towards personalized medicine. BJU international. 2012; 109: 1864-70. doi:10.1111/j.1464-410X.2011.10661.x.

23. Kroog GS, Motzer RJ. Systemic therapy for metastatic renal cell carcinoma. The Urologic clinics of North America. 2008; 35: 687-701; ix. doi:10.1016/j.ucl.2008.07.007.

24. Zigeuner R, Hutterer G, Chromecki T, Imamovic A, Kampel-Kettner K, Rehak P, et al. External validation of the Mayo Clinic stage, size, grade, and necrosis (SSIGN) score for clear-cell renal cell carcinoma in a single European centre applying routine pathology. European urology. 2010; 57: 102-9. doi:10.1016/j.eururo.2008.11.033.

25. Gulati S, Martinez P, Joshi T, Birkbak NJ, Santos CR, Rowan AJ, et al. Systematic evaluation of the prognostic impact and intratumour heterogeneity of clear cell renal cell carcinoma biomarkers. European urology. 2014; 66: 936-48. doi:10.1016/j.eururo.2014.06.053.

26. Fisel P, Kruck S, Winter S, Bedke J, Hennenlotter J, Nies AT, et al. DNA methylation of the SLC16A3 promoter regulates expression of the human lactate transporter MCT4 in renal cancer with consequences for clinical outcome. Clinical cancer research : an official journal of the American Association for Cancer Research. 2013; 19: 5170-81. doi:10.1158/1078-0432.CCR-13-1180.

27. Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S. Occludin: a novel integral membrane protein localizing at tight junctions. The Journal of cell biology. 1993; 123: 1777-88.

28. Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. The Journal of cell biology. 1996; 134: 1031-49.

29. Balda MS, Flores-Maldonado C, Cereijido M, Matter K. Multiple domains of occludin are involved in the regulation of paracellular permeability. Journal of cellular biochemistry. 2000; 78: 85-96.

30. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nature reviews Molecular cell biology. 2001; 2: 285-93. doi:10.1038/35067088.

31. Farquhar MG, Palade GE. Junctional complexes in various epithelia. The Journal of cell biology. 1963; 17: 375-412.

32. Orban E, Szabo E, Lotz G, Kupcsulik P, Paska C, Schaff Z, et al. Different expression of occludin and ZO-1 in primary and metastatic liver tumors. Pathology oncology research : POR. 2008; 14: 299-306. doi:10.1007/s12253-008-9031-2.

33. Martin TA, Mansel RE, Jiang WG. Loss of occludin leads to the progression of human breast cancer. International journal of molecular medicine. 2010; 26: 723-34.

34. Tobioka H, Isomura H, Kokai Y, Tokunaga Y, Yamaguchi J, Sawada N. Occludin expression decreases with the progression of human endometrial carcinoma. Human pathology. 2004; 35: 159-64.

35. Tobioka H, Tokunaga Y, Isomura H, Kokai Y, Yamaguchi J, Sawada N. Expression of occludin, a tight-junction-associated protein, in human lung carcinomas. Virchows Archiv : an international journal of pathology. 2004; 445: 472-6. doi:10.1007/s00428-004-1054-9.

36. Rachow S, Zorn-Kruppa M, Ohnemus U, Kirschner N, Vidal-y-Sy S, von den Driesch P, et al. Occludin is involved in adhesion, apoptosis, differentiation and Ca2+-homeostasis of human keratinocytes: implications for tumorigenesis. PloS one. 2013; 8: e55116. doi:10.1371/journal.pone.0055116.

37. Osanai M, Murata M, Nishikiori N, Chiba H, Kojima T, Sawada N. Epigenetic silencing of occludin promotes tumorigenic and metastatic properties of cancer cells via modulations of unique sets of apoptosis-associated genes. Cancer research. 2006; 66: 9125-33. doi:10.1158/0008-5472.CAN-06-1864.

38. Ellinger J, Kahl P, Mertens C, Rogenhofer S, Hauser S, Hartmann W, et al. Prognostic relevance of global histone H3 lysine 4 (H3K4) methylation in renal cell carcinoma. International journal of cancer Journal international du cancer. 2010; 127: 2360-6. doi:10.1002/ijc.25250.

39. Ye D, Guo S, Al-Sadi R, Ma TY. MicroRNA regulation of intestinal epithelial tight junction permeability. Gastroenterology. 2011; 141: 1323-33. doi:10.1053/j.gastro.2011.07.005.

40. Munari E, Marchionni L, Chitre A, Hayashi M, Martignoni G, Brunelli M, et al. Clear cell papillary renal cell carcinoma: micro-RNA expression profiling and comparison with clear cell renal cell carcinoma and papillary renal cell carcinoma. Human pathology. 2014; 45: 1130-8. doi:10.1016/j.humpath.2014.01.013.

41. White NM, Bao TT, Grigull J, Youssef YM, Girgis A, Diamandis M, et al. miRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. The Journal of urology. 2011; 186: 1077-83. doi:10.1016/j.juro.2011.04.110.

42. Zhou L, Chen J, Li Z, Li X, Hu X, Huang Y, et al. Integrated profiling of microRNAs and mRNAs: microRNAs located on Xq27.3 associate with clear cell renal cell carcinoma. PloS one. 2010; 5: e15224. doi:10.1371/journal.pone.0015224.

43. Fong MY, Zhou W, Liu L, Alontaga AY, Chandra M, Ashby J, et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. Nature cell biology. 2015; 17: 183-94. doi:10.1038/ncb3094.

44. Ruaro ME, Stebel M, Vatta P, Marzinotto S, Schneider C. Analysis of the domain requirement in Gas1 growth suppressing activity. FEBS letters. 2000; 481: 159-63.

45. Stebel M, Vatta P, Ruaro ME, Del Sal G, Parton RG, Schneider C. The growth suppressing gas1 product is a GPI-linked protein. FEBS letters. 2000; 481: 152-8.

46. Del Sal G, Ruaro ME, Philipson L, Schneider C. The growth arrest-specific gene, gas1, is involved in growth suppression. Cell. 1992; 70: 595-607.

47. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature. 2004; 432: 324-31. doi:10.1038/nature03100.

48. Allen BL, Tenzen T, McMahon AP. The Hedgehog-binding proteins Gas1 and Cdo cooperate to positively regulate Shh signaling during mouse development. Genes & development. 2007; 21: 1244-57. doi:10.1101/gad.1543607.

49. Katoh Y, Katoh M. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review). International journal of molecular medicine. 2008; 22: 271-5.

50. Wang H, Zhou X, Zhang Y, Zhu H, Zhao L, Fan L, et al. Growth arrest-specific gene 1 is downregulated and inhibits tumor growth in gastric cancer. The FEBS journal. 2012; 279: 3652-64. doi:10.1111/j.1742-4658.2012.08726.x.

51. Jiang Z, Xu Y, Cai S. Down-regulated GAS1 expression correlates with recurrence in stage II and III colorectal cancer. Human pathology. 2011; 42: 361-8. doi:10.1016/j.humpath.2010.03.009.

52. Gobeil S, Zhu X, Doillon CJ, Green MR. A genome-wide shRNA screen identifies GAS1 as a novel melanoma metastasis suppressor gene. Genes & development. 2008; 22: 2932-40. doi:10.1101/gad.1714608.

53. Scaltriti M, Brausi M, Amorosi A, Caporali A, D'Arca D, Astancolle S, et al. Clusterin (SGP-2, ApoJ) expression is downregulated in low- and high-grade human prostate cancer. International journal of cancer Journal international du cancer. 2004; 108: 23-30. doi:10.1002/ijc.11496.

54. Sacilotto N, Espert A, Castillo J, Franco L, Lopez-Rodas G. Epigenetic transcriptional regulation of the growth arrest-specific gene 1 (Gas1) in hepatic cell

proliferation at mononucleosomal resolution. PloS one. 2011; 6: e23318. doi:10.1371/journal.pone.0023318.

55. Ma Y, Qin H, Cui Y. MiR-34a targets GAS1 to promote cell proliferation and inhibit apoptosis in papillary thyroid carcinoma via PI3K/Akt/Bad pathway. Biochemical and biophysical research communications. 2013; 441: 958-63. doi:10.1016/j.bbrc.2013.11.010.

56. Zhang L, He S, Guo S, Xie W, Xin R, Yu H, et al. Down-regulation of miR-34a alleviates mesangial proliferation in vitro and glomerular hypertrophy in early diabetic nephropathy mice by targeting GAS1. Journal of diabetes and its complications. 2014; 28: 259-64. doi:10.1016/j.jdiacomp.2014.01.002.

57. Liu H, Brannon AR, Reddy AR, Alexe G, Seiler MW, Arreola A, et al. Identifying mRNA targets of microRNA dysregulated in cancer: with application to clear cell Renal Cell Carcinoma. BMC systems biology. 2010; 4: 51. doi:10.1186/1752-0509-4-51.

58. Yu G, Li H, Wang J, Gumireddy K, Li A, Yao W, et al. miRNA-34a suppresses cell proliferation and metastasis by targeting CD44 in human renal carcinoma cells. The Journal of urology. 2014; 192: 1229-37. doi:10.1016/j.juro.2014.05.094.

59. Dutta KK, Zhong Y, Liu YT, Yamada T, Akatsuka S, Hu Q, et al. Association of microRNA-34a overexpression with proliferation is cell type-dependent. Cancer science. 2007; 98: 1845-52. doi:10.1111/j.1349-7006.2007.00619.x.

60. Yan S, Wang Y, Chen M, Li G, Fan J. Deregulated SLC2A1 Promotes Tumor Cell Proliferation and Metastasis in Gastric Cancer. International journal of molecular sciences. 2015; 16: 16144-57. doi:10.3390/ijms160716144.

61. Amann T, Maegdefrau U, Hartmann A, Agaimy A, Marienhagen J, Weiss TS, et al. GLUT1 expression is increased in hepatocellular carcinoma and promotes tumorigenesis. The American journal of pathology. 2009; 174: 1544-52. doi:10.2353/ajpath.2009.080596.

62. Krzeslak A, Wojcik-Krowiranda K, Forma E, Jozwiak P, Romanowicz H, Bienkiewicz A, et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. Pathology oncology research : POR. 2012; 18: 721-8. doi:10.1007/s12253-012-9500-5.

63. Manel N, Kim FJ, Kinet S, Taylor N, Sitbon M, Battini JL. The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV. Cell. 2003; 115: 449-59.

64. Augustin R. The protein family of glucose transport facilitators: It's not only about glucose after all. IUBMB life. 2010; 62: 315-33. doi:10.1002/iub.315.

65. Cifuentes M, Garcia MA, Arrabal PM, Martinez F, Yanez MJ, Jara N, et al. Insulin regulates GLUT1-mediated glucose transport in MG-63 human osteosarcoma cells. Journal of cellular physiology. 2011; 226: 1425-32. doi:10.1002/jcp.22668.

66. Vermeulen JF, van Brussel AS, van der Groep P, Morsink FH, Bult P, van der Wall E, et al. Immunophenotyping invasive breast cancer: paving the road for molecular imaging. BMC cancer. 2012; 12: 240. doi:10.1186/1471-2407-12-240.

67. Vermeulen JF, van der Wall E, Witkamp AJ, van Diest PJ. Analysis of expression of membrane-bound tumor markers in ductal carcinoma in situ of the breast: paving the way for molecular imaging. Cell Oncol (Dordr). 2013; 36: 333-40. doi:10.1007/s13402-013-0138-4.

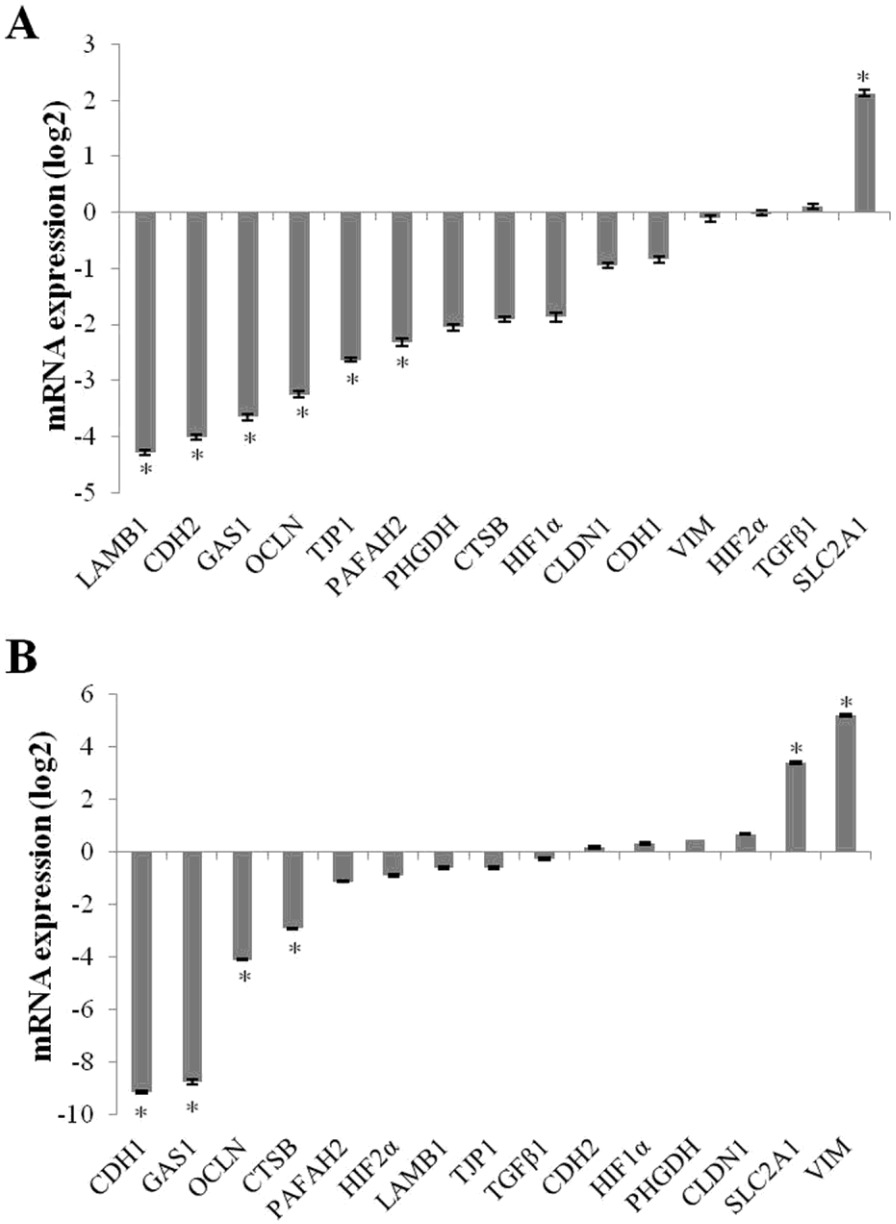

Figure 1. Gene expression profile in ccRCC. mRNA expression of the selected genes using qPCR in ccRCC samples (A) and in 786-O cell line (B). The results are shown as the fold change in expression relative to normal sample (*$p < 0.001$, Wilcoxon Signed Rank Test).

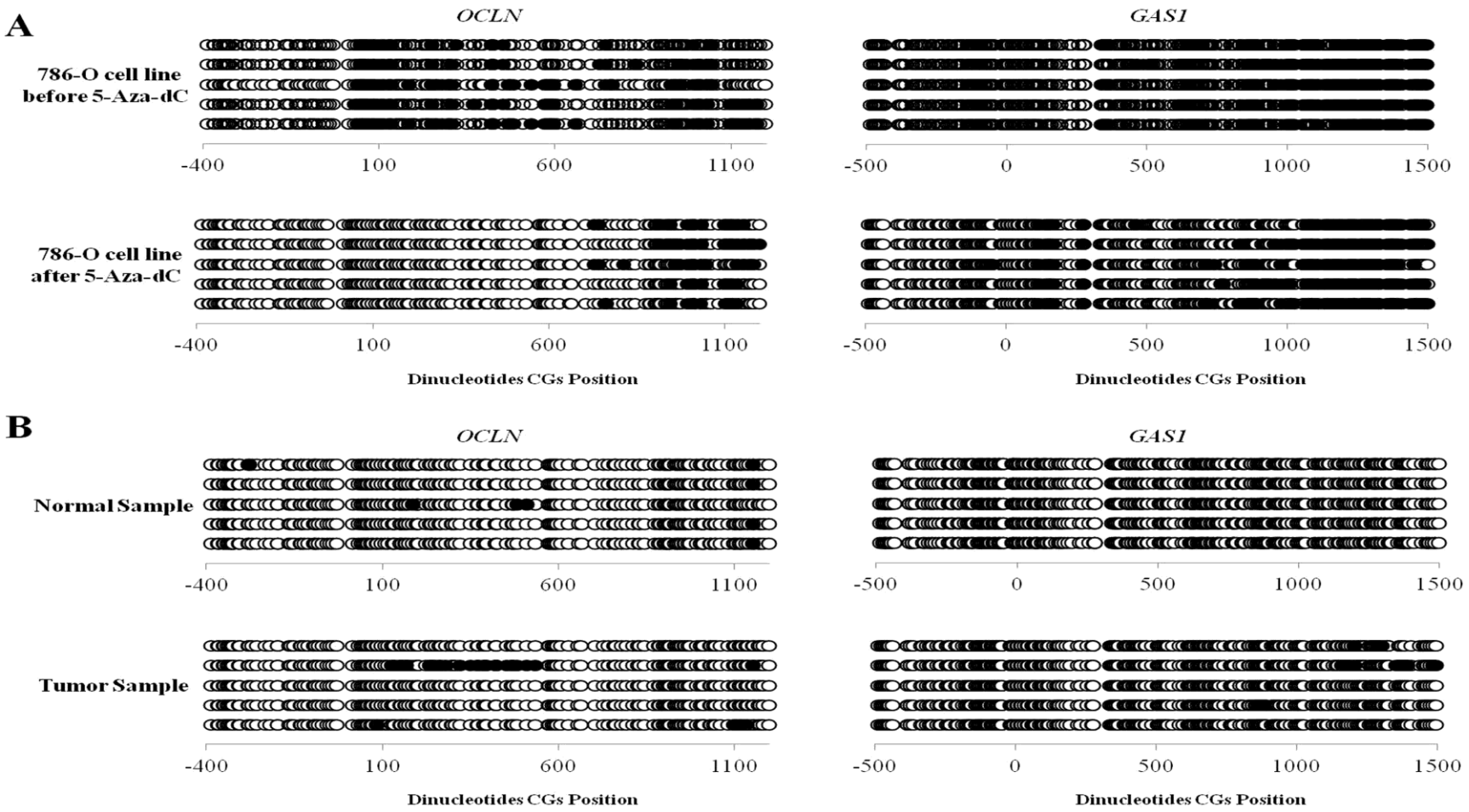

Figure 2. Methylation level in *OCLN* and *GAS1* gene promoters. The transcription initiation site is represented by +1. Expanded view shows the position of CpG islands and region analyzed by bisulfite sequencing. Each row represents one sequenced clone, and open filled circles represent unmethylated CpG dinucleotides, respectively. (A) Bisulfite sequencing of *OCLN* and *GAS1* before and after 5-Aza-dC treatment, (B) Bisulfite sequencing of *OCLN* and *GAS1* in normal and tumor sample.

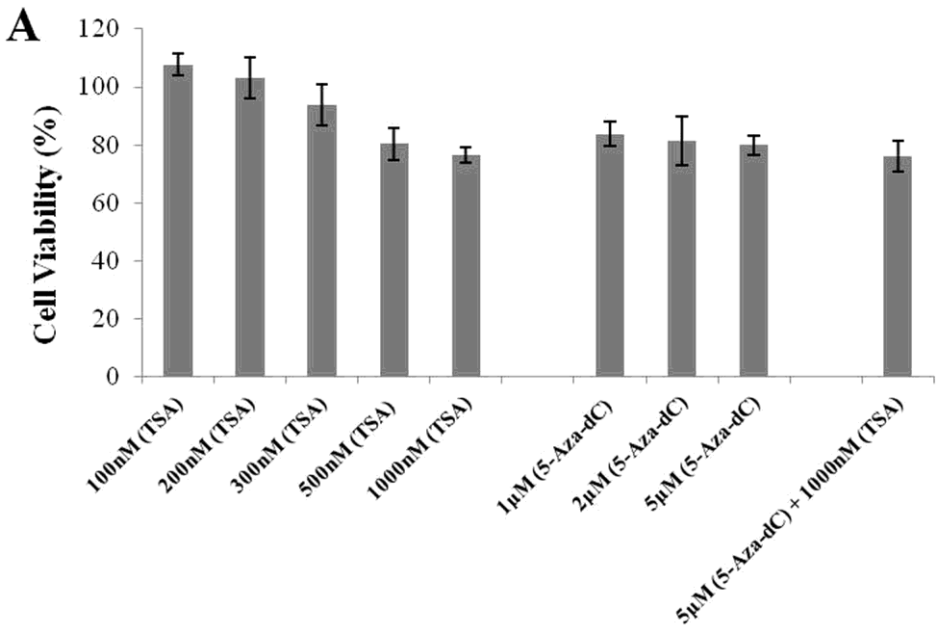

Figure 3. 786-O cell viability after 5-Aza-dC and TSA treatment. Cell viability of 786-O cells after 5-Aza-dC, TSA and co-treatment with 5-Aza-dC and TSA.

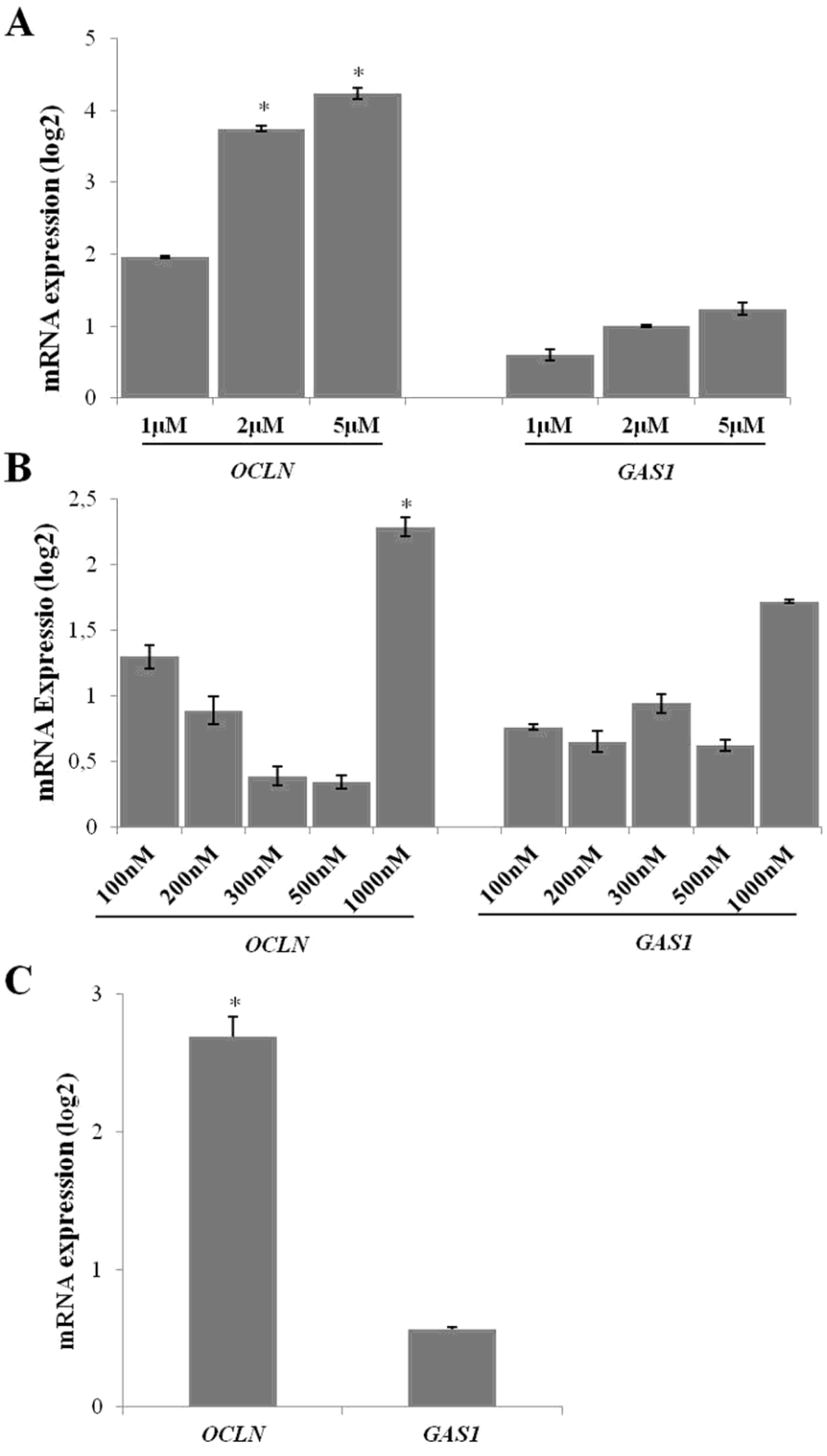

Figure 4. *OCLN* and *GAS1* expression level after 5-Aza-dC and TSA treatment. (A) mRNA expression of *OCLN* and *GAS1* gene in 786-O cell line after 5-Aza-dC treatment. (B) mRNA expression of *OCLN* and *GAS1* gene in 786-O cell line after TSA treatment, (C) mRNA expression of *OCLN* and *GAS1* in 786-O cell line after co-treatment with 5-Aza-dC and TSA. (*p < 0.001, Wilcoxon Signed Rank Test)

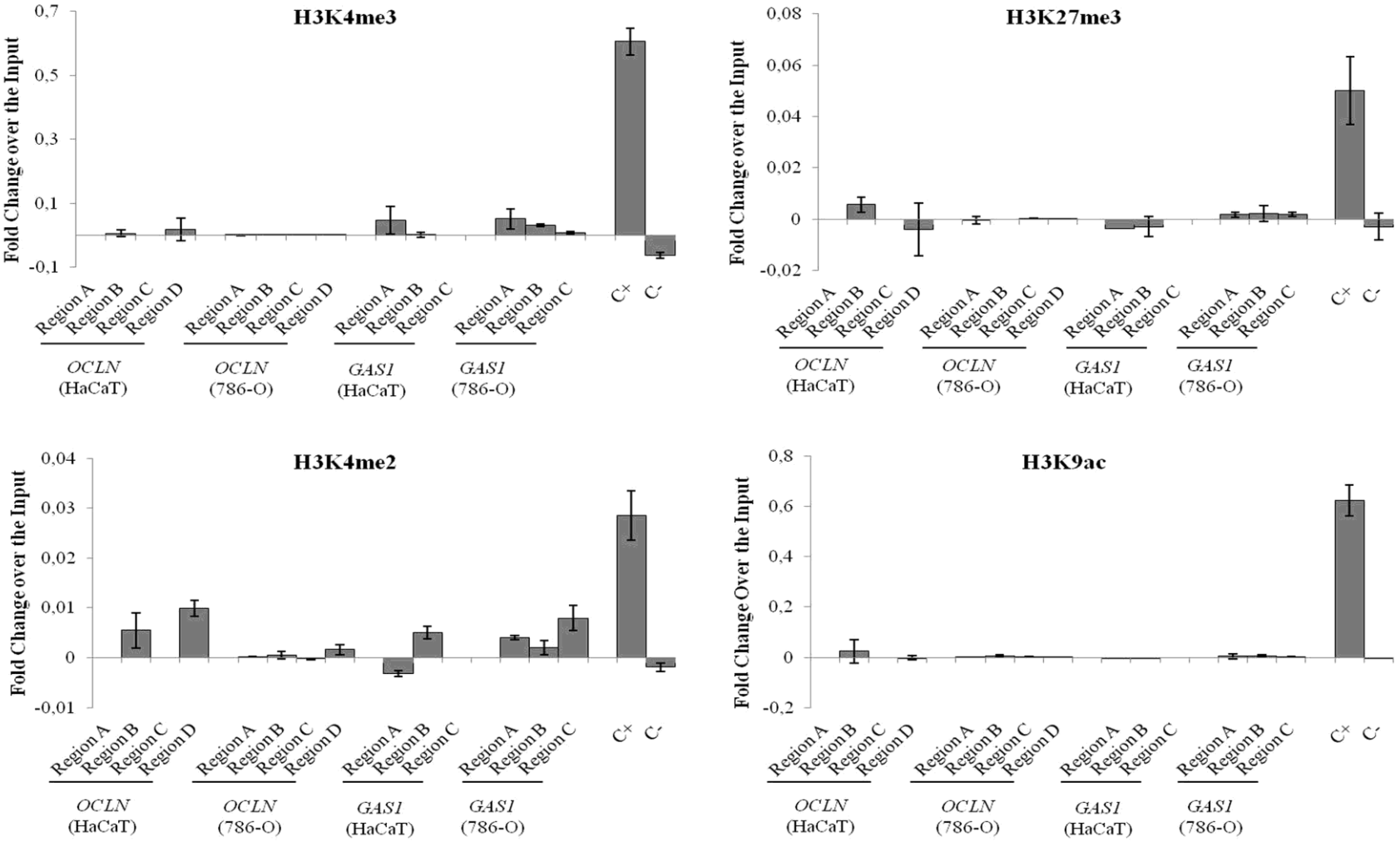

Figure 5. Histone modifications in *OCLN* and *GAS1* gene promoters. ChIP analyses of the levels of H3K4me3, H3K27me3, H3K4me2 and H3K9ac in the *OCLN* and *GAS1* promoter regions. The values are represented as fold change over the input.

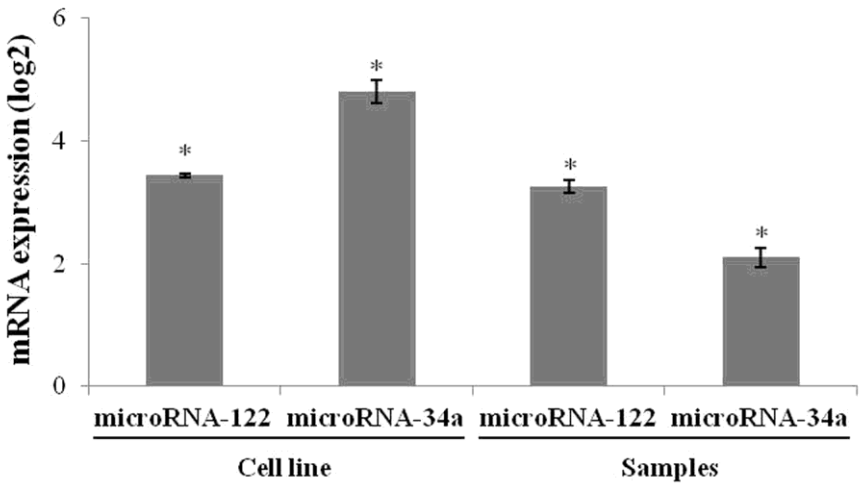

Figure 6. MicroRNA-122 and microRNA-34a expression are upregulated in ccRCC. miR-122 and miR-34a expression levels in 786-O cell line compared with HaCaT cell line and ccRCC samples compared with normal renal tissue samples. (*$p < 0.001$, Wilcoxon Signed Rank Test).

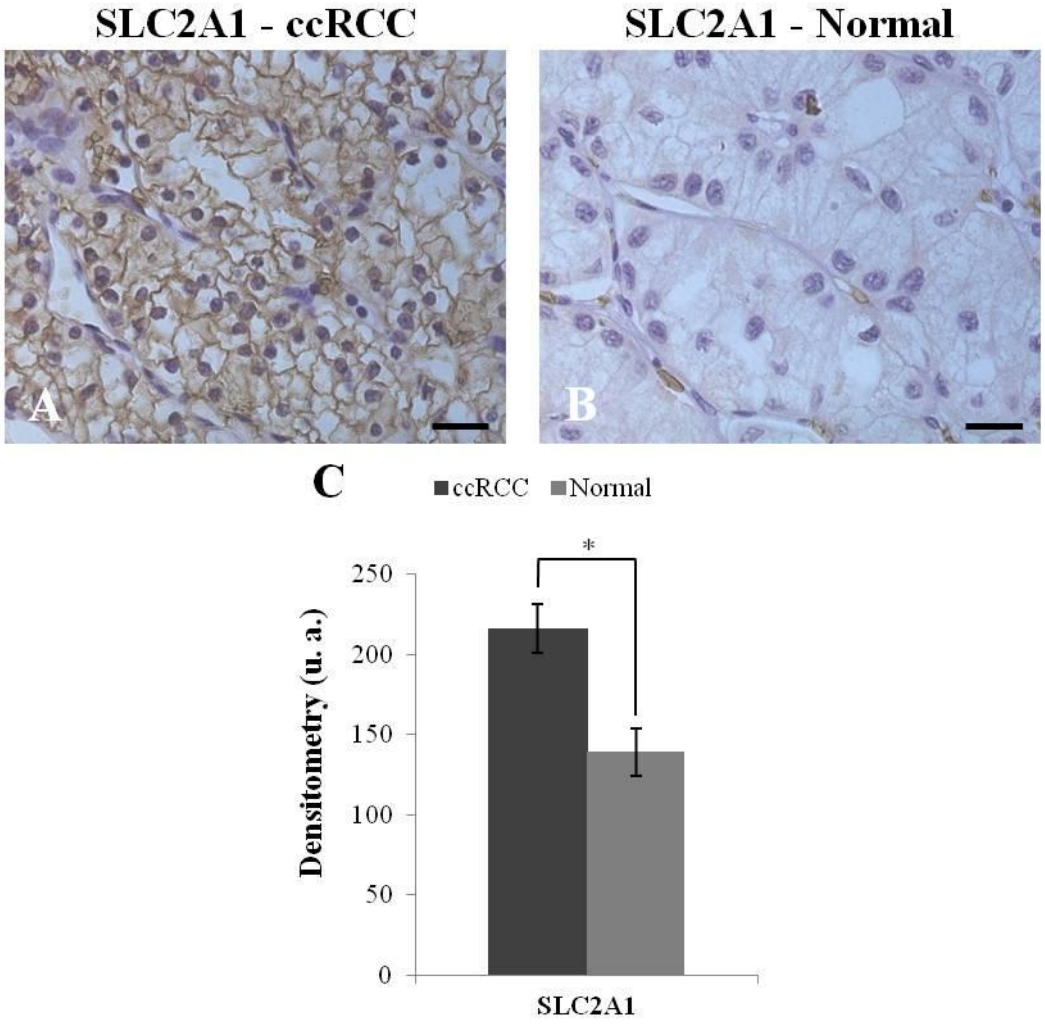

Figure 7. SLC2A1 is highly expressed in ccRCC compared to normal samples. Protein detection of (A) SLC2A1 in ccRCC sample, (B) SLC2A1 in normal sample, (C) Graphic of densitometry of the immunostaining of SLC2A1 in the samples analyzed. Bars = 20 μm. (*$p < 0.0001$, Mann-Whitney test).

Table S1. Primers sequences used in the quantitative real-time polymerase chain reaction and bisulfite sequencing.

Gene	Primer Forward	Primer Reverse
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCAAATTCGT
CTSB	GGAGTGTACCAACACGTCACC	CATTGTCACCCCAGTCAGTG
CLDN1	GGTGCAGAAGATGAGGATGG	CATTGACTGGGGTCATAGGG
GAS1	GAAGGGATGGTTGGGGATAC	GGGAGGAGGTCCAAGAAGTC
SLC2A1	GTCTGGCATCAACGCTGTC	AGCGACACGACAGTGAAGG
HIF2A	TGGTAGCCCTCTCCAACAAG	ACCCCTGAGGTTCTTCATCC
PHGDH	TTGCTGTTCAGTTCGTGGAC	TGTGTGGAGAGAAGGCACTG
TGFβ1	ATTGAGGGCTTTCGCCTTAG	GTAGTGAACCCGTTGATGTCC
TJP1	AGGTGAAACACTGCTGAGTCC	AGTGTGGTAAGCGCAGCTC
PAFAH2	AGTTCAGCTCCGGCAAGTC	AAGCTCCCCTGGAGATTCTG
LAMB1	AGTGGAAGGAATGGTTCACG	TTTTACAGGCGTTGCTGTTTC
CDH2	ATATGGCCTTTCAAACACAGC	CGTCATGGCAGTAAACTCTGG
OCLN	CCCGTTTGGATAAAGAATTGG	CTGCAGATCCCTTCACTTGC
VIM	GAAATTGCAGGAGGAGATGC	ATTCCACTTTGCGTTCAAGG
CDH1	GAACGCATTGCCACATACAC	ATTCGGGCTTGTTGTCATTC
OCLN – Seq.1	CCTCTCTCATCAGACACCCC	CTCCTTTCCCTCCTCCCTACAAC
OCLN – Seq.2	GTTGTAGGGAGGAGGGAAAGGAG	TATCCAAAGGGGTGGGGTGG
OCLN – Seq.3	CCACCCCACCCCTTTGG	ACACTGACTGGCCTGGAAAGC
GAS1 – Seq.1	AACAGGCAGGACCAATAGCATCT	GCTCCTTCCTCCTGGGCTCA
GAS1 – Seq.2	TGAGCCCAGGAGGAAGGAGC	GCTCCCCCTGGCACTGCA
GAS1 – Seq.3	GCAGTGCCAGGGGGAGC	CCTTGGGCATAGCCAGCAT
GAS1 – Seq.4	TCATTGAGGACATGCTGGCTATG	CCCTTCTATCTGTCCCAAGCCAC
***GAS1* – Reg.A**	GGACCAGATCTCGACAGCTG	TTCATAAATCCATGCGCGCC
***GAS1* – Reg.B**	CAAACAGCTGCCCCTAAGC	GCTCACATTCAGGCATTCAG
***GAS1* – Reg.C**	CGAGAAGGCAGAGGTTTCC	TGTCGAGCAATTACCACCTG
***OCLN* – Reg.A**	TTGGAAGCTAAAGGGCATTG	ATCAGGCCTGTAAGGAGGTG
***OCLN* – Reg.B**	CACCATGCTCCCACTTCAC	TAGACCAGGAGAGCCTGTGG
***OCLN* – Reg.C**	ATGGAGGGTCGTTTTGAGG	GTGTGAGGCAACAATGAAGG
***OCLN* – Reg.D**	GCGACTATGTTAGGGGAGGTG	CTCTCTGGTTCCCACGAGTC

Table S2. Primers sequences used in quantitative real time polymerase chain reaction for microRNAs.

microRNA	Target Gene	Primer Sequences (5'- 3')
		F – ACACTCCAGCTGGGTGGCAGTGTCTTAGCTGG
miR-34a	*GAS1*	R – TGGTGTCGTGGAGTCG
		RT - CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACAACCAG
		F – ACACTCCAGCTGGGTGGAGTGTGACAATGGTGTTTG
miR-122	*OCLN*	R – CTCAACTGGTGTCGTGGA
		RT - CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAACAC
		F – TCTGAGTGTCTTCGCTGACG
U48		R – GAGGTATTCGCACCAGAGGA
		RT – GTCTCCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGGAGACGGTCAG

CONCLUSÕES

CONCLUSÕES

O presente trabalho permitiu as seguintes conclusões:

1. Os genes *OCLN* e *GAS1* apresentam uma redução de expressão significativa em amostras de ccRCC e em linhagem celular de carcinoma renal, quando comparada com a amostra renal normal;

2. Tanto a metilação do DNA quanto as modificações de histonas analisadas não atuam no processo de regulação dos genes *OCLN* e *GAS1*;

3. Os microRNAs miR-122 e miR-34a apresentam um aumento de expressão significativa em amostras de ccRCC e em linhagem celular de carcinoma renal, quando comparada com a amostra renal normal e, possivelmente estão regulando a expressão dos genes *OCLN* e *GAS1*, respectivamente;

4. O gene e a proteína SLC2A1 apresentam um aumento de expressão significativa em amostras de ccRCC e em linhagem celular de carcinoma renal, quando comparada com a amostra renal normal.

REFERÊNCIAS

REFERÊNCIAS

ACS. American Cancer Society. Disponível em www.cancer.org. Acesso em: 19 jan. 2016.

AIJAZ, S.; BALDA, M. S.; MATTER, K. Tight junctions: molecular architecture and function. **Int Rev Cytol**, v. 248, p. 261-298, 2006.

AMANN, T. et al. GLUT1 expression is increased in hepatocellular carcinoma and promotes tumorigenesis. **Am J Pathol**, v. 174, p. 1544-1552, 2009.

ARAI, E. et al. Single-CpG-resolution methylome analysis identifies clinicopathologically aggressive CpG island methylator phenotype clear cell renal cell carcinomas. **Carcinogenesis**, v. 33, p. 1487-1493, 2012.

AUWERA I. V. et al. The presence of circulating total DNA and methylated genes is associated with circulating tumour cells in blood from breast cancer patients. **Br J Cancer**, v. 100, p. 1277-1286, 2009.

BANDI, N.; VASSELLA, E. miR-34a and miR-15a/16 are co-regulated in non-small cell lung cancer and control cell cycle progression in a synergistic and Rb-dependent manner. **Mol Cancer**, v. 10, p. 1-11, 2011.

BAO, B. et al. Over-expression of *FoxM1* leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. **J Cell Biochem**, v. 112, p. 2296-2306, 2011.

BAYLIN, S. B. DNA methylation and gene silencing in cancer. **Nat Clin Pract Oncol**, v. 2, p. 4-11, 2005.

BAYLIN, S. B.; OHM, J. E. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? **Nat Rev Cancer**, v. 6, p. 107-116, 2006.

BEISLAND, C. et al Multiple primary malignancies in patients with renal cell carcinoma: a national population-based cohort study. **BJU Int**, v. 97, p. 698-702, 2006.

BENARD, A. et al. Histone trimethylation at H3K4, H3K9 and H4K20 correlates with patient survival and tumor recurrence in early-stage colon cancer. **BMC cancer**. v. 14, p. 531, 2014.

CALIN, G. A.; CROECE, C. M. MicroRNA signatures in human cancer. **Nat Rev Cancer**, v. 6, p. 857-866, 2006.

CATTO, J. W. et al. MicroRNA in prostate, bladder, and kidney cancer: a systematic review. **Eur Urol**, v. 59, p. 671-681, 2011.

CHO, W. C. MicroRNAs: potential biomarkers for cancer diagnosis, prognosis and targets for therapy. **Int J Biochem Cell Biol**, v. 42, p. 1273-1281, 2010.

CIFUENTES, M., et al. Insulin regulates GLUT1-mediated glucose transport in MG-63 human osteosarcoma cells. **J Cell Physiol**, v. 226, p. 1425-1432, 2011.

COHEN, H. T. MCGOVERN, F. J. Renal-cell carcinoma. **N Engl J Med**, v. 353, p. 2477-2490, 2005.

COOPER, G. M. **The molecular cell approach**. Ed. Sinauer. 2. ed., 2000.

COTTRELL, S. E. Molecular diagnostic applications of DNA methylation technology. **Clin Biochem**, v. 37, p. 595-604, 2004.

CRAENE, B. D.; BERX, G. Regulatory networks defining EMT during cancer initiation and progression. **Nat Rev Cancer**, v. 13, p. 97-110, 2013.

DOMINGUEZ-MONZON, G. et al. Gas1 inhibits cell proliferation and induces apoptosis of human primary gliomas in the absence of Shh. **Int J Dev Neurosci**, v. 27, p. 305-313, 2009.

DAWSON, M. A.; KOUZARIDES, T. Cancer epigenetics: from mechanism to therapy. **Cell**, v. 150, p. 12-27, 2012.

DRUCKER, B. J. Renal cell carcinoma: Current status and future prospects. **Cancer Treat Rev**, v. 31, p. 536-545, 2005.

DU, T.; ZAMORE, P. D. microPrimer: the biogenesis and function of microRNA. **Development**, v. 132, 4645-4652, 2005.

EGGER, G. et al. Epigenetics in human disease and prospects for epigenetic therapy. **Nature**, v. 429, p. 457-463, 2004.

ELLINGER, J. et al. Prognostic relevance of global histone H3 lysine 4 (H3K4) methylation in renal cell carcinoma. **Int J Cancer**, v. 127, p. 2360-2366, 2010.

EVDOKIOU, A.; COWLED, P. A. Tumor-suppressive activity of the growth arrest-specific gene GAS1 in human tumor cell lines. **Int J Cancer**, v. 75, p. 568-577, 1998.

EZPONDA, T.; LICHT, J. D. Molecular pathways: deregulation of histone h3 lysine 27 methylation in cancer-different paths, same destination. **Clin Cancer Res**, v. 20, 5001-5008, 2014.

FEINBERG, A. P.; TYCKO, B. The history of cancer epigenetics. **Nat Rev Cancer**, v. 4, p. 143-153, 2004.

FILIPOWICZ, W. RNAi: the nuts and bolts of the RISC machine. **Cell**, v. 122, 17-20, 2005.

FINLEY, D. S.; PANTUCK, A. J.; BELLDEGRUN, A. S. Tumor biology and prognostic factors in renal cell carcinoma. **Oncologist**, v. 16, p. 4-13, 2011.

FONG, M. Y. et al. Breast-cancer-secreted miR122 reprograms glucose metabolism in premetastatic niche to promote metastasis. **Nat Cell Biol**, v. 17, p. 183-196, 2015.

FRITZSCHE F. R. et al. Claudin-1 protein expression is a prognostic marker of patient survival in renal cell carcinomas. **Clin Cancer Res**, v. 14, p. 7035-7042, 2008.

GARZON, R..; MARCUCCI, G. Potential of microRNAs for cancer diagnostics, prognostication and therapy. **Curr Opin Oncol**, v. 24, p. 655-659, 2012.

GIRARD, M. et al. miR-122, a paradigm for the role of microRNAs in the liver. **J Hepatol**, v. 48, p. 648-656, 2008.

GREGORY, R. I. et al. The Microprocessor complex mediates the genesis of microRNAs. **Nature**, v. 432, 235-240, 2004.

GUTTILLA, I. K. et al. Prolonged mammosphere culture of MCF-7 cells induces an EMT and repression of the estrogen receptor by microRNAs. **Breast Cancer Res Treat**, v. 132, p. 75-85, 2011.

HARTEN, S. K. et al. Regulation of renal epithelial tight junctions by the von Hippel-Lindau tumor suppressor gene involves occludin and claudin 1 and is independent of E-cadherin. **Mol Biol Cell**, v. 20, p. 1089-1101, 2009.

HERMAN, J. G.; BAYLIN, S. B. Gene silencing in cancer in association with promoter hypermethylation. **N Engl J Med**, v. 349, p. 2042- 2054, 2003.

HERTIG, A.; FLIER, S. N.; KALLURI R. Contribution of Epithelial Plasticity to Renal Transplantation–Associated Fibrosis. **Transplant Proc**, v. 42, p. 7-12, 2010.

HOLLANDE, F. et al. Adherens junctions and tight junctions are regulated via different pathways by progastrin in epithelial cells. **J Cell Sci**, v. 116, p. 1187-1197, 2002.

HSU, S. H. et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. **J Clin Invest**, v. 122, p. 2871-2883, 2012.

HSU, S. H.; GHOSHAL, K. MicroRNAs in Liver Health and Disease. **Curr Pathobiol Rep**, v. 1, p. 53-62, 2013.

HUANG, T. T. et al. Epigenetic deregulation of the anaplastic lymphoma kinase gene modulates mesenchymal characteristics of oral squamous cell carcinomas. **Carcinogenesis**. v. 34, p. 1717-1727, 2013.

IARC. Inernational Agency for Research on Cancer. Disponível em www.iarc.fr. Acesso em 19 jan. 2016.

IKENOUCHI, J. et al. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. **J Cell Sci**, v. 116, p. 1959-1967, 2003.

INCA. Instituto Nacional do Câncer José Alencar Gomes da Silva. Disponível em www.inca.org.br. Acesso em: 14 dez. 2015.

INCA. Instituto Nacional do Câncer José Alencar Gomes da Silva. Disponível em www.inca.org.br. Acesso em 10 jan. 2016.

ISERI, O. D. et al. Drug resistant MCF-7 cells exhibit epithelial-mesenchymal transition gene expression pattern. **Biomed Pharmacother**, v. 65, p. 40-45, 2011.

ISHIGAKI, Y. et al. Scanning Electron Microscopy With an Ionic Liquid Reveals the Loss of Mitotic Protrusions of Cells During the Epithelial–Mesenchymal Transition. **Microsc Res Tech**, v. 74, p. 1024-1031, 2011.

JANG, M. J.; BAEK, S. H.; KIM, J. H. UCH-L1 promotes cancer metastasis in prostate cancer cells through EMT induction. **Cancer Letters**, v. 302, p. 128-135, 2011.

JIANG Z et al. Down-regulated GAS1 expression correlates with recurrence in stage II and III colorectal cancer. **Hum Pathol**, v. 42, p. 361-368, 2011.

JIN, J-C. et al. MicroRNA‑122 regulation of the morphology and cytoarchitecture of hepatoma carcinoma cells. **Mol Med Rep**, v. 9, p. 1376-1380, 2014.

JONES, P. A. Functions of DNA methylation: islands, start sites, gene bodies and beyond. **Nat Ver Genet**, v. 13, p. 484-492, 2012.

JONES, P. A.; BAYLIN, S. B. The Epigenomics of Cancer. **Cell**, v. 128, p. 683-692, 2007.

JOPLING C. Liver-specific microRNA-122: Biogenesis and function. **RNA Biol**, v. 9, p. 137-142, 2012.

KARAGIANNIS, G. S. et al. Collective migration of cancer-associated fibroblasts is enhanced by overexpression of tight junction-associated proteins claudin-11 and occluding. **Mol Oncol**, p. 1-18, 2013.

KATOH, Y.; KATOH, M. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA. **Int J Mol Med**, v. 22, p. 271-275, 2008.

KAWAMURA, T. et al. Expression of glucose transporter-1 in human gastric carcinoma: Association with tumor aggressiveness, metastasis, and patient survival. **Cancer**, v. 92, p. 634–641, 2001.

KE, A. W. et al. CD151 Amplifies signaling by integrin α6β1 to PI3K and induces the epithelial-mesenchymal transition in HCC cells. **Gastroenterology**, v. 140, p. 1629-1641, 2011.

KHAKPOUR, G. et al. DNA methylation as a promising landscape: A simple blood test for breast cancer prediction. **Tumour Biol**, v. 36, p. 4905-4912, 2015.

KIM, T. et al. p53 regulates epithelial–mesenchymal transition through microRNAs targeting ZEB1 and ZEB2. **J Exp Med**, v. 208, p. 875-883, 2011.

KIM, V. N. MicroRNA Biogenesis: Coordinated Cropping and Dicing**. Nat Rev Mol Cell Biol**, v. 6, p. 376-386, 2005.

KRZESLAK, A. et al. Expression of GLUT1 and GLUT3 glucose transporters in endometrial and breast cancers. **Pathol Oncol Res**, v. 18, p. 721–728, 2012.

KURODA, N. et al. Clear cell renal cell carcinoma with focal renal angiomyoadenomatous tumor-like area. **Ann Diagn Pathol**, v. 15, p. 202-206, 2011 .

LABER, D. A. Risk Factores, Classification and Staging of Renal Cell Cancer. **Med Oncol**, v. 23, p. 443-454, 2006.

LEE, Y. et al. The nuclear RNase III drosha initiates microRNA processing. **Nature**, v. 425, 415-419, 2003.

LEWIS, B. P.; BURGE, C. B.; BARTEL, D. P. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. **Cell**, v. 120, p. 15-20, 2005.

LI, J.; MANSMANN, U. R. A microRNA molecular modeling extension for prediction of colorectal cancer treatment. **BMC cancer**, v. 15, p. 472, 2015.

LI, X. et al. D9S168 Microsatellite Alteration Predicts a Poor Prognosis in Patients With Clear Cell Renal Cell Carcinoma and Correlates With the Down-Regulation of Protein Tyrosine Phosphatase Receptor Delta. **Cancer**, v. 117, p. 4201-4211, 2011.

LIPWORTH, L.; TARONE, R. E.; MCLAUGHLIN, J. K. The Epidemiology of Renal Cell Carcinoma. **J Urol**, v. 176, p. 2353-2358, 2006.

LIU, Y. Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. **J Am Soc Nephrol**, v. 15, p. 1-12, 2004.

LIU, Y. New Insights into Epithelial-Mesenchymal Transition in Kidney Fibrosis. **J Am Soc Nephrol**, v. 21, p. 212-222, 2010.

LIU, W.; MAO, S .Y.; ZHU, W. Y. Impact of tiny miRNAs on cancers. **Wourld Journal of Gastroenterology**, v. 28, p. 497-502, 2007.

LODISH, H. et al. **Molecular Cell Biology**, 4. ed. Ed. W. H. Freeman, Nova Iorque, 2000.

LOPEZ-BELTRAN A. et al. 2004 WHO classification of renal tumours of the adults. **Eur Urol**, v. 49, p. 798-805, 2006.

LOPEZ-BELTRAN, A. et al. 2009 update on the classification of renal epithelial tumors in adults. **International Journal of Urology**, v. 16, p. 432-443, 2009.

MA, Y.; QIN, H.; CUI, Y. MiR-34a targets GAS1 to promoted cell proliferation and inhibit apoptosis in papillary thyroid carcinoma via PI3K/Akt/Bad pathway. **Biochem Biophys Res Commun**, v. 441, p. 958-963, 2013.

MALLORY, A. C.; VAUCHERET, H. MicroRNAs: something important between the genes. **Current Opinion in Plant Biology**, v. 67, p. 120-125, 2004.

MOES, M. et al., A novel network integrating a mirna-203/snai1 feedback loop which regulates epithelial to mesenchymal transition. **Plos One**, v. 7, p. 1-7, 2012.

MOSASHVILLI, D. et al. Global histone acetylation levels: prognostic relevance in patients with renal cell carcinoma. **Cancer Sci**, v. 101, p. 2664-2669, 2010.

MUNARI, E. et al. Clear cell papillary renal cell carcinoma: micro-RNA expression profiling and comparison with clear cell renal cell carcinoma and papillary renal cell carcinoma. **Hum Pathol**, v. 45, p. 1130-1138, 2014.

NAGATA, M. et al. Aberrations of a cell adhesion molecule CADM4 in renal clear cell carcinoma. **Int J Cancer**, v. 130, p. 1329-1337, 2012.

NALLS, D. et al. Targeting epigenetic regulation of miR-34a for treatment of pancreatic cancer by inhibition of pancreatic cancer stem cells. **Plos One**, v. 6, p. 1-12, 2011.

NELSON. E. C.; EVANS, C. P.; LARA, P. N. Renal cell carcinoma: Current status and emerging therapies. **Cancer Treatment Reviews**, v. 33, p.299-313, 2007.

NOGUCHI, Y. et al. Suppression of facilitative glucose transporter 1 mRNA can suppress tumor growth. **Cancer Lett.**, v. 154, p. 175–182, 2000.

NOH, H. et al. Histone deacetylase-2 is a key regulator of diabetes and transforming growth factor-beta1-induced renal injury. **Am J Physiol Renal Physiol**, v. 297, p. 729-739, 2009.

PAIVA, F. et al. Functional and epigenetic characterization of the KRT19 gene in renal cell neoplasms. **DNA and Cell Biology**, v. 30, p. 85-90, 2011.

PERIER, A. et al. Mutations of the *von Hippel–Lindau* gene confer increased susceptibility to natural killer cells of clear-cell renal cell carcinoma. **Oncogene**, v. 30, p. 2622-2633, 2011.

PETRELLA, B. L.; BRINCKERHOFF, C. E. Tumor cell invasion of von Hippel Lindau renal cell carcinoma cells is mediated by membrane type-1 matrix metalloproteinase. **Mol Cancer**, v. 5, p. 66-80, 2006.

PIERUCCI-ALVES, F. et. al. Transforming Growth Factor Beta 1 Induces Tight Junction Disruptions and Loss of Transepithelial Resistance Across Porcine Vas Deferens Epithelial Cells. **Bio Reprod**, v. 86, p. 1-8, 2011.

RACHOW, S. et al. Occludin Is Involved in Adhesion, Apoptosis, Differentiation and Ca2+ - Homeostasis of Human Keratinocytes: Implications for Tumorigenesis. **PLoS ONE**. v. 8, p. 1-13, 2013.

RATHOD, S. S. et al. Tumor suppressive miRNA-34a suppresses cell proliferation and tumor growth of glioma stem cells by targeting Akt and Wnt signaling pathways. **FEBS Open Bio**, v. 4, p. 485-495, 2014.

RAUCH, T.; PFEIFER, G. P. Methylated-CpG islands recovery assay: a new technique for the rapid detection of methylated-CpG islands in cancer. **Lab Invest**, v. 85, p. 1172-1180, 2005.

REDOVA, M.; SVOBODA, M.; SLABY, O. MicroRNAs and their target gene networks in renal cell carcinoma. **Biochem Biophys Res Commun**, v. 405, p. 153-156, 2011.

RODRÍGUEZ-RODERO, S. et al. DNA methylation signatures identify biologically distinct thyroid cancer subtypes. **J Clin Endocrinol Metab**, v. 98, p. 2811-2821, 2013.

ROGENHOFER, S. et al. Global histone H3 lysine 27 (H3K27) methylation levels and their prognostic relevance in renal cell carcinoma. **BJU Int**, v. 109, p. 459-465, 2012.

RU, P. et al. miRNA-29b suppresses prostate cancer metastasis by regulating epithelial-mesenchymal transition signaling. **Mol Cancer Ther**, v. 11, p. 1166-1173, 2012.

SAETROM, P.; SNOVE JR, O.; ROSSI, J. J. Epigenetics and microRNAs. **Pediatr Res**, v. 61, p. 17-23, 2007.

SANDRASEGARAN, K. et al. Usefulness of diffusion-weighted imaging in the evaluation of renal masses. **AJR Am J Roentgenol**, v. 194, p. 438-445, 2010.

SELIGSON, D. B. et al. Global levels of histone modifications predict prognosis in different cancers. **Am J Pathol**, v. 174, p. 1619-1628, 2009.

SENDI, H. et al. miR-122 decreases HCV entry into hepatocytes through binding to the 3' UTR of OCLN mRNA. **Liver int**, v. 35, p. 1315-1323, 2015.

SEPULVEDA, J.L. et al. High-definition CpG methylation of novel genes in gastric carcinogenesis identified by next-generation sequencing. **Mod Pathol**, v. 29, p. 182-93, 2016.

SHOOK, D.; KELLER, R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. **Mech Dev**, v. 120, p. 1351-83, 2003.

SIM, J. et al. High MicroRNA-370 Expression Correlates with Tumor Progression and Poor Prognosis in Breast Cancer. **J Breast Cancer**, v. 18, p. 323-328, 2015.

STEBEL, M.; VATTA, P.; RUARO, M. E.; DEL SAL, G.; PARTON, R. G.; SCHNEIDER, C. The growth suppressing gas1 product is a GPI-linked protein. **FEBS Lett**, v. 481, p. 152-158, 2000.

SULEWSKA, A. et al. DNA methylation in states of cell physiology and pathology. **Folia histochem Cytobiol**, v. 45, p. 149-158, 2007.

SZABO, G.; BALA, S. MicroRNAs in liver disease. **Nat Rev Gastroenterol Hepatol**, v. 10, p. 542-552, 2013.

SHUCH, B. et al. Understanding pathologic variants of renal cell carcinoma: distilling therapeutic opportunities from biologic complexity. **Eur Urol**, v. 67, p. 85-97, 2015.

TAKAHASHI, M. etal. Gene expression profiling of clear cell renal cell carcinoma: gene identification and prognostic classification. **Proc Natl Acad Sci USA**, v. 98, p.9754-9759, 2001.

TANG, J.; ZHUANG, S. Epigenetics in acute kidney injury. **Curr Opin Nephrol Hypertens**, v. 24, p. 351-358, 2015.

TAYLOR, M. A. et al. Enhanced MAPK signaling drives ETS1-mediated induction of miR-29b leading to downregulation of TET1 and changes in epigenetic modifications in a subset of lung SCC. **Oncogene**, p. 1-13, 2016.

THIERY, J. P.; SLEEMAN, J. P. Complex networks orchestrate epithelial-mesenchymal transitions. **Nat Rev Mol Cell Biol**, v. 7, p. 131-142, 2006.

TIWARI, N. et al. EMT as the ultimate survival mechanism of cancer cells. **Semin Cancer Biol**, v. 22, p. 194-207, 2012.

TSAI, W. C. et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. **J Clin Invest**, v. 122, p. 2884-2897, 2012.

TSUJI, T.; IBARAGI, S.; HU, G. F. Epithelial-mesenchymal transition and cell cooperativity in metastasis. **Cancer research**, v. 69, 7135-7139, 2009.

TUN, H. W. et al. Pathway Signature and Cellular Differentiation in Clear Cell Renal Cell Carcinoma. **PLoS One**, v. 5, p. 1-14, 2010.

USHIJIMA, T. Detection and interpretation of altered methylation patterns in cancer cells. **Nat Rev Cancer**, v. 5, p. 223-231, 2005.

VERMEULEN, J. F. et al. Immunophenotyping invasive breast cancer paving the for molecular imaging. **BMC Cancer**, v. 12, p. 1-10, 2012.

VERMEULEN, J. F.; van der WALL, E.; WITKAMP, A. J.; DIEST, P. J. Analysis of expression of membrane-bound tumor markers in ductal carcinoma in situ of breast: paving the way for molecular imaging. **Cell Oncol**, v. 36, p. 333-340, 2013.

VERVERIS, K. et al. V. Histone deacetylase inhibitors (HDACIs): multitargeted anticancer agents. **Biologics**, v. 7, p. 47-60, 2013.

VISVADER, J. E. Cells of origin in cancer. **Nature**, v. 469, p. 314-322, 2011.

WACHI, S.; YONEDA, K.; WU, R. Interactome-transcriptome analysis reveals the high centrality of genes differentially expressed in lung cancer tissues. **Bioinformatics**, v. 21, p. 4205-4208, 2005.

WALSH, L. A.; NAWSHAD, A.; MEDICI, D. Discoidin domain receptor 2 is a critical regulator of epithelial–mesenchymal transition. **Matrix Biol**, v. 30, p. 243-247, 2011.

WANG H. et al. Growth arrest-specific gene 1 is downregulated and inhibits tumor growth in gastric cancer. **FEBS J**, v. 279, p. 3652-3664, 2012.

WANG, L. Q. et al. Epigenetic silencing of tumor suppressor miR-3151 contributes to Chinese chronic lymphocytic leukemia by constitutive activation of MADD/ERK and PIK3R2/AKT signaling pathways. **Oncotarget**, v. 6, p. 44422-44436, 2015.

WANG, L. J. et al. Hypermethylation of testis derived transcript gene promoter significantly correlates with worse outcomes inglioblastoma patients. **Chin Med J**, v. 126, p. 2062-2066, 2013.

WANG, Z. et al. MicroRNA-663 suppresses cell invasion and migration by targeting transforming growth factor beta 1 in papillarythyroid carcinoma. **Tumour Biol**, p. 1-12, 2015.

WCRFI. World Cancer Research Fund International. Disponível em www.wcrf.org. Acesso em: 19 jan. 2016.

WENG, W. et al. YY1-C/EBPα-miR34a regulatory circuitry is involved in renal cell carcinoma progression. **Oncol Rep**, v. 31, p. 1921-1927, 2014.

WHITE N. M. et al. miRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. **J Urol**, v. 186, p. 1077-1083, 2011.

WU, J. et al. HAb18G/CD147 promotes epithelial–mesenchymal transition through TGF-b signaling and is transcriptionally regulated by Slug. **Oncogene**, v. 30, p. 4410-4427, 2011.

WU, X. et al. De novo sequencing of circulating miRNAs identifies novel markers predicting clinical outcome of locally advanced breast cancer. **J Transl Med**, v. 10, p. 1-10, 2012.

WU, Y-Y. et al. SCUBE3 is an endogenous TGF-b receptor ligand and regulates the epithelial-mesenchymal transition in lung cancer. **Oncogene**, v. 30, p. 3682-3693, 2011.

YAN, S. et al. Deregulated SLC2A1 promotes tumor cell proliferation and metastasis in gastric câncer. **Int J Mol Sci**, v. 16, p. 16144-16157, 2015.

YE, D. et al. MicroRNA regulation of intestinal epithelial tight junction permeability. **Gastroenterology**, v. 141, p. 1323-1333, 2011.

ZARCO, N. et al. GAS1 induces cell death through an intrinsic apoptotic pathway. **Apoptosis**, v. 17, p. 627-635, 2012.

ZBAR, B. et al. Familial Renal Carcinoma: Clinical Evaluation, Clinical Subtypes and Risk of Renal Carcinoma Development. **J Urol**, v. 177, p. 461-465, 2007.

ZENG, Y. Principles of micro-RNA production and maturation. **Oncogene**, v. 25, p. 6156-6162, 2006.

ZHANG, J. P. et al. MicroRNA-148a suppresses the epithelial-mesenchymal transition and metastasis of hepatoma cells by targeting Met/Snail signaling. **Oncogene**, v. 33, p. 4069-4076, 2014.

ZHANG, L. et al. Down regulation of miR-34a alleviates mesangial proliferation in vitro and glomerular hypertrophy in early diabetic nephropathy mice by targeting GAS1. **J Diabetes Complications**, v. 28, p. 259-264, 2014.

ZHANG, W.; DAHLBERG, J. E.; TAM, W. MicroRNAs in tumorigenesis: a primer. **Am J Pathol**, v. 171, p. 728-738, 2007.

ZHANG, X. et al. The significance of microRNA-184 on JAK2/STAT3 signaling pathway in the formation mechanism ofglioblastoma. **Oncol Lett**, v. 10, p. 3510-3514, 2015.

ZHAO, L.; WANG, W. miR-125b suppresses the proliferation of hepatocellular carcinoma cells by targeting Sirtuin7. **Int J Clin Med**, v. 8, p. 18469-18475, 2015.

ZHI, M. L. et al. Diagnostic performance of microRNA-29a for colorectal cancer: a meta-analysis. **Genet Mol Res**, v. 14, p. 18018-18025, 2015.

ZHOU, L. et al. Integrated profiling of microRNAs and mRNAs: MicroRNAs located on Xq27.3 associate with clear cell carcinoma. **PLoS One**, v. 5, p. 1-10, 2010.

ZHOU, Z. et al. Inhibition of the growth of non-small cell lung cancer by miRNA-1271. **Am J Transl Res**, v. 7, p. 1917-1914, 2015.