UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

AVALIAÇÃO DA RESPOSTA *IN VITRO* AO TRATAMENTO COM INIBIDOR DE RECEPTOR TIROSINA QUINASE (PALLADIA ®) E mTORC1 (RAPAMICINA) EM CÉLULAS DE CARCINOMA PROSTÁTICO CANINO

PRISCILA EMIKO KOBAYASHI

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Tese apresentada junto
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Carcinoma prostático (CaP)

Fator de crescimento do endotélio vascular (VEGF)

Receptor do fator de crescimento do endotélio vascular (VEGFR)

Fator de crescimento derivado de plaquetas (PDGF)

Receptor do fator de crescimento derivado de plaquetas (PDGFR)

SCF (fator de célula tronco)

Instituto Nacional do Câncer (INCA)

mTOR (proteína alvo da rapamicina em mamíferos)

mTORC1 (complexo 1 da proteína alvo da rapamicina em mamíferos)

Adenosina trifosfato (ATP)

Tumor gastrointestinal (GIST)

Receptor de fator de crescimento epidérmico 1 (EGFR ou HER 1)

Receptor de fator de crescimento epidérmico 2 (HER 2)

Receptor de fator de crescimento epidérmico 4 (HER 4)

Fator de crescimento epidérmico (EGF)

Dióxido de carbono (CO2)

Proteína alvo da rapamicina (mTOR)

Complexo 1 da mTOR (mTORC1)

Complexo 2 da mTOR (mTORC2)

Fator de iniciação eucariótico 4E proteína de ligação 1 (4E-BP1)

Fator de iniciação eucariótico 4E (eIF-4E)

Proteína quinase ribossomal S6 (S6Ks)

fosfatidilinositol-3 -quinase (PI3K)

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SUMÁRIO

KOBAYASHI P.E. **Avaliação da resposta in vitro ao tratamento com inibidor de receptor tirosina quinase (palladia ®) e mtorc1 (rapamicina) em células de carcinoma prostático canino**. Botucatu, 2020, 106 p. Tese (Doutorado) _ Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

RESUMO

O carcinoma prostático canino possui prognóstico desfavorável e modalidades terapêuticas limitadas, principalmente em casos avançados. Devido à falta de tratamento eficaz para os carcinomas prostáticos caninos, esse estudo objetivou avaliar o efeito do toceranib (inibidor de receptor tirosina quinase) e da rapamicina (inibidor de mTORC1) em células de carcinoma prostático canino. As duas células de cultura primária advindas de carcinoma prostático canino, denominadas PC1 e PC2, apresentaram expressão proteica de VEGFR2 e PDGFR-β e ausência da proteína c-KIT, os quais são alvos do fosfato de toceranib. Adicionalmente, após comparação do transcriptoma das células não tratadas e tratadas com fosfato de toceranib, observaram-se alterações em genes relacionados com a via PDGFR, angiogênese e resistência terapêutica. Além disso, realizou-se o tratamento das células PC1 e PC2 com rapamicina (inibidor de mTORC1) após a comparação do transcriptoma das células tumorais com células de próstata normal e identificação de genes envolvidos na via PI3K/AKT/mTOR. O tratamento com rapamicina diminuiu a expressão gênica de *mTOR* e *4E-BP1* e aumentou de *AKT*, podendo representar mecanismos de resistência. Após tratamento com fosfato de toceranib, apenas a PC1 reduziu a viabilidade celular, porém após tratamento com rapamicina, as células PC1 e PC2 reduziram a viabilidade celular de maneira dose dependente, havendo resposta biológica em ambas as terapias. Sendo assim, o bloqueio de múltiplos receptores tirosina quinase e inibição de mTOR representam alvos terapêuticos no carcinoma prostático canino, entretanto a resistência terapêutica ainda é desafiadora e, portanto, a medicina de precisão seria uma forma de superar o problema.

Palavras-chave: VEGFR, PDGFR, c-KIT, mTOR, transcriptoma

KOBAYASHI P.E. ***In vitro*** **evaluation of receptor tyrosine kinase inhibitor (Palladia®) and mTORC1 inhibitor (rapamycin) in canine prostatic carcinoma cells.** Botucatu, 2020, 106 p. Tese (Doutorado) _ Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

Canine prostatic carcinoma has a poor prognosis and limited therapeutic modalities, mainly in advanced cases. The aim of this study was to evaluate the effect of toceranib phosphate (receptor tyrosine kinase inhibitor) and rapamycin (mTORC1 inhibitor) in canine prostatic carcinoma cells due to the lack of efficient therapies. Two primary canine prostate cancer cell lines, PC1 and PC2, had protein expression of VEGFR2 and PDGFR-β and absent c-KIT expression, which are target toceranib phosphate targets. Additionally, we compared transcriptome before and after toceranib phosphate treatment and we observed gene alterations related to PDGFR pathway, angiogenesis and therapeutic resistance. Besides, we treated PC1 and PC2 cells with rapamycin after transcriptome analysis comparing canine prostatic cells from normal prostate and neoplastic cells and identification of genes involved in PI3K/AKT/mTOR pathway. Gene expression of *mTOR* and *4E-BP1* decreased and *AKT* increased after rapamycin treatment, and could represent a resistency mechanism. A decrease only in PC1 cell viability was observed after toceranib phosphate treatment, however decreased cell viability in a dose-dependent manner was observed in PC1 and PC2 cells after rapamycin treatment. Therefore, we could observe biological response of canine prostatic carcinoma cells to both therapies. Thus, multiple receptor tyrosine kinase inhibition and mTOR inhibition represent target therapies to canine prostatic carcinoma. However, therapeutic resistance still remains a challenge and precision medicine could be a way to overcome this problem.

Key- words: VEGFR, PDGFR, c-KIT, m-TOR, transcriptome

Capítulo 1
INTRODUÇÃO

Segundo dados do Instituto Nacional do Câncer (INCA), o câncer de próstata no homem será o mais incidente durante o ano de 2020, com exceção do câncer de pele não melanoma, com 65.840 novos casos, correspondendo a 29,2% de todas as neoplasias (INCA, 2019). Em cães, embora não existam dados retrospectivos atuais sobre prevalência, é reportado baixa prevalência de carcinoma prostático (CaP) canino em material provindo de necropsia (WEAVER, 1981).

Dentre os grandes mamíferos, o cão é o único que possui desenvolvimento espontâneo da neoplasia prostática, além de lesões prostáticas e sítios de metástases similares ao humano (osso, linfonodos, pulmão), tornando-o um importante modelo de estudo em neoplasias prostáticas (CORNELL et al., 2000; GUPTA; MASSAGUÉ, 2006; HENSEL; THALMANN, 2016; PINHO et al., 2012; WEAVER, 1981). Outra escolha para novos modelos de estudo é o uso do cultivo celular, como por exemplo, a utilização de linhagens celulares de neoplasias que tem proporcionado descobertas relacionadas à carcinogênese e metástases (ITOU et al., 2015). Além disso, o inicio de desenvolvimento da maioria dos agentes antineoplásicos dependem de testes *in vitro,* pois o baixo custo e a rápida avaliação permitem uma triagem e desenvolvimento rápido de novos agentes terapêuticos (GORDON; KHANNA, 2010), associado a minimização de problemas relacionados a questões éticas com o uso dos modelos *in vitro* (KRAMER et al., 2013).

O câncer de próstata é um complexo multifatorial, e apresenta grande importância devido a sua alta morbidade e mortalidade (KELLER et al., 2013; LEROY; NORTHRUP, 2009). Dentre os fatores que colaboram para essa estatística é a presença de metástase, com debilitação e complicações progressivas (ARYA et al., 2006). A metástase no carcinoma prostático é um mecanismo em que envolve processos múltiplos das células e do microambiente tumoral, incluindo a angiogênese, proliferação e invasão celular (ARYA et al., 2006; GUPTA; MASSAGUÉ, 2006).

Os eventos do início da carcinogênese até sua metástase são processos dinâmicos que envolvem vias de sinalização entre as células neoplásicas e imunes, estroma e vasos (LONG et al., 2014). A transição epitelial-mesenquimal participa em um desses processos, para que as células percam a polaridade e adesão celular, ganhem propriedades invasivas e migrem para outros locais (FONSECA-ALVES et al., 2015; KOBAYASHI et al., 2018).

Em humanos, apesar da melhoria dos métodos diagnósticos precoce, ainda ocorrem mortes devido à neoplasia prostática e sua progressão (WOZNEY; ANTONARAKIS, 2014). Portanto, apesar de o câncer prostático apresentar comportamento de crescimento lento, trata-se de uma doença potencialmente fatal com o avanço da neoplasia, havendo grande interesse no desenvolvimento de tratamentos com menor toxicidade e, consequentemente melhor qualidade de vida durante os anos (HACKSHAW-MCGEAGH et al., 2015).

Em cães, geralmente o CaP apresenta um prognóstico reservado, pois geralmente é diagnosticado em estágio avançado e com alta taxa de metástase, dificultando o tratamento (LEROY; NORTHRUP, 2009). Uma das causas no diagnóstico tardio é a ausência de sinais clínico ou também sinais clínicos não patognomônicos que incluem hematúria, estrangúria e tenesmo (CORNELL et al., 2000; SUN; BÁEZ-DÍAZ; SÁNCHEZ-MARGALLO, 2017). Portanto, para o diagnóstico definitivo ser estabelecido também são necessários: exame físico, diagnóstico por imagem, exames citopatológico e histopatológico (BENNETT et al., 2018). Os tratamentos incluem terapias locais (prostatectomia, radioterapia, por exemplo) e sistêmicas (inibidor de ciclooxigenase, quimioterapia), porém sem uma taxa de sobrevida satisfatória (LEROY; NORTHRUP, 2009; SORENMO et al., 2004). Sendo assim, novas terapias também são necessárias nessa espécie e dentre elas a terapia alvo, a qual o objetivo é destruir as células neoplásicas ou antígenos associados, sem afetar as células normais (HOJJAT-FARSANGI, 2014).

O tratamento do câncer geralmente está correlacionado com tratamentos que atuam em alvos moleculares comuns para células normais e neoplásicas, dessa forma a pesquisa de novas terapias que interfiram com alvos intracelulares envolvidos na carcinogênese estão em progresso e são

necessárias para possível uso combinado com terapias convencionais (VICENTINI et al., 2003).

O Fosfato de Toceranib (Palladia®) é um medicamento inibidor de receptores de tirosina quinase, os quais possuem papel na proliferação e sobrevivência celular, além de ativação de células endoteliais para neovascularização (RANIERI et al., 2013). Os inibidores de tirosina quinase podem representar uma estratégia adicional de tratamento, atingindo a proliferação celular e metástase, reduzindo a morbidade associada aos carcinomas prostáticos e suas metástases (OJEMUYIWA; MADAN; DAHUT, 2014). Além disso, estudo *in vitro* com osteossarcoma canino reduziu a migração celular e após inoculação dessas células de cultura em camundongos, esses animais foram tratados e apresentou redução de crescimento celular *in vivo* (SÁNCHEZ-CÉSPEDES et al., 2019).

O uso do Palladia® foi aprovado na Medicina Veterinária e sua eficácia é sabidamente comprovada em casos de mastocitoma canino, porém o seu uso em outras neoplasias pode ser viável devido às alterações dos receptores alvos nos diferentes tumores (CHON et al., 2012; LEBLANC et al., 2012). Foram demonstradas respostas clínicas na fase I do medicamento em cães com diversos tipos de neoplasias com desenvolvimento espontâneo, incluindo melanoma, mieloma múltiplo, diferentes carcinomas e sarcomas, inclusive em alguns casos com metástases (LONDON et al., 2003). Além disso, tem sido avaliado o uso conjunto de Palladia® e outra medicação, dentre eles o piroxicam, doxorrubicina e vimblastina (CHON et al., 2012; PELLIN et al., 2017; ROBAT et al., 2012). A associação do fosfato de toceranib ao piroxicam em cães com carcinoma prostático demonstrou resposta parcial ao tratamento (PAN et al., 2014) ou estabilização da neoplasia (CHON et al., 2012).

Outro medicamento utilizado em terapia antineoplásica é a rapamicina, a qual inibe o mTORC1 (complexo 1 da proteína alvo da rapamicina em mamíferos), bloqueando assim a via de progressão do ciclo celular, proliferação e angiogênese (LARSON et al., 2016). Em humanos, a via PI3K/AKT/mTOR apresenta papel importante no desenvolvimento do CaP (RUSSO et al., 2019). Em cães, as proteínas dessa via têm sido avaliada em diferentes neoplasias, incluindo hemangiossarcoma (MURAI et al., 2012a), osteossarcoma (GORDON; YE; KENT, 2008a), melanoma (KENT; COLLINS;

YE, 2009) e, inclusive, em carcinoma prostático (RIVERA-CALDERÓN et al., 2019a). Diante disso, observa-se a alteração da via PI3K/AKT/ mTOR em diferentes neoplasias e podem apresentar possíveis alvo terapêuticos no tratamento antineoplásico em cães.

Portanto, diante da problemática que o câncer prostático representa na espécie canina e humana, associado à falta de alternativas no tratamento com alta eficácia dependendo do estágio clínico da doença, fazem-se necessários maiores estudos com novas terapias a fim de uma melhoria clínica para o paciente.

REVISÃO DE LITERATURA

1. PRÓSTATA CANINA

Em cães machos, a próstata é a única glândula sexual e localiza-se na cavidade pélvica ou abdômen caudal dependendo do tamanho, cranialmente a bexiga e envolve a uretra proximal (KUTZELER & YEAGER, 2005; EVANS & CHRISTENSEN, 1993). Apresenta formato oval a esférica, com um sulco dorsal e ventral, recoberta por cápsula fibromuscular (SMITH, 2008).

Além disso, a próstata canina é uma glândula serosa e sofre alteração histológica durante a maturação sexual do animal, como por exemplo, em glândulas imaturas a porção acinar é pouco desenvolvida, lúmen pequeno e sem evidência de atividade secretória (DORSO et al., 2008). Com o desenvolvimento da próstata, o lúmen das glândulas tubuloalveolares aumenta e, consequentemente, o estroma do tecido conjuntivo diminui (DORSO et al., 2008). Essas alterações são decorrentes da influência de andrógenos durante o desenvolvimento e crescimento de cães não castrados. E em casos de neoplasias prostáticas caninas, os andrógenos parecem ter efeito protetor (TESKE et al., 2002).

As células basais epiteliais prostáticas são apresentam-se de forma descontínua e são pouco visíveis na coloração de hematoxilina e eosina (FONSECA-ALVES et al., 2015; LEAV et al., 2001).

2. NEOPLASIA PROSTÁTICA E SUA METÁSTASE

O câncer de próstata é um grande problema de saúde mundial e, de acordo com a GLOBOCAN, estimou-se 1,3 milhões de novos casos de câncer

de próstata no mundo e 359 mil associados a mortes para o ano de 2018 (BRAY et al., 2018). Nos Estados Unidos, a neoplasia prostática lidera o ranking do número de novos casos de câncer no homem (SIEGEL; MILLER; JEMAL, 2018). No Brasil, esta liderança é similar, com previsão de aproximadamente 66 mil novos casos para o ano de 2020 (INCA, 2019).

Apesar da alta morbidade e mortalidade decorrente da neoplasia prostática, a etiologia do câncer prostático ainda é desconhecida, porém são citados e estudados diversos fatores de risco em humanos: idade, raça, dieta (gordura e carne vermelha), obesidade e histórico prévio familiar (BASHIR, 2015; BOSTWICK et al., 2004; HSING; DEVESA, 2001).

Assim como em outras neoplasias, o câncer prostático desenvolve-se através de alterações genéticas somáticas e epigenéticas (DE MARZO et al., 2007), além de inflamação e proliferação celular aumentada, os quais são alguns dos fatores que podem iniciar uma cascata de eventos que conduzem a lesões prostáticas, incluindo neoplasia (JOSHI et al., 2015).

A sinalização do andrógeno e seu receptor têm papel importante durante o desenvolvimento da glândula prostática humana, bem como na carcinogênese prostática e progressão, podendo tornar-se letal quando a neoplasia é independente de andrógeno (BANERJEE et al., 2018; TINDALL; LONERGAN, 2011). Em cães, observou-se perda do receptor de andrógeno nos carcinomas prostáticos em relação às próstatas normais, o que torna possível a utilização desses animais como modelo experimental de carcinoma prostático humano independente de andrógeno (RIVERA-CALDERÓN et al., 2016).

Para a iniciação e crescimento de células epiteliais, incluindo do carcinoma prostático, a proliferação celular excessiva e a angiogênese são etapas cruciais (KALLURI; WEINBERG, 2009). Porém, uma das etapas que determina um pior prognóstico em um paciente com carcinoma prostático é a aquisição da capacidade celular de invadir e migrar (VAN DE MERBEL et al., 2018). Em cães e no homem, há tendência para metástase esquelética durante o desenvolvimento espontâneo da neoplasia prostática, sendo que o tratamento em casos de metástases são apenas paliativos (KELLER et al., 2013; WIESNER et al., 2008).

Durante cada etapa na cascata da metástase, as células tumorais interagem com o microambiente e seus componentes (GANAPATHY; MOGHE; ROTH, 2015). As propriedades invasivas e migratórias são primordiais durante o processo de metástase, portanto são necessárias enzimas que participam na degradação da membrana basal, além de reorganização do citoesqueleto celular, perda de polaridade e adesão (KALLURI; WEINBERG, 2009; KHAN et al., 2015). Algumas dessas alterações estão presentes durante o processo de transição epitelial-mesenquimal que é caracterizado pela obtenção das células epiteliais de um fenótipo invasivo e mesenquimal que permite a migração celular para novos microambientes (WEI et al., 2008). Em cães e humanos, essa transição tem sido estudada como um evento importante para que o carcinoma prostático metastatize (FONSECA-ALVES et al., 2015; KHAN et al., 2015). Durante essa etapa as células tumorais apresentam aumento em sua motilidade, favorecendo a disseminação para locais distantes, porém há uma diminuição da capacidade proliferativa. Entretanto, o processo inverso pode ocorrer: as células com fenótipo mesenquimal adquirem novamente as características epiteliais, inclusive com maior capacidade de proliferação (LI; LI, 2015; MARCUCCI; STASSI; DE MARIA, 2016).

Devido à importância das neoplasias prostáticas nos cães e humanos e os diversos mecanismos envolvidos na carcinogênese e sua metástase, diferentes técnicas são necessárias para que se obtenha maiores informações a respeito dessa doença e suas terapias. Diante disso, a cultura celular é uma ferramenta que é utilizada em descobertas e testes de terapias diversas doenças, inclusive o carcinoma prostático humano (ELLEM; DE-JUAN-PARDO; RISBRIDGER, 2014). Assim como todo modelo experimental, a cultura celular em monocamada apresenta vantagens e desvantagens. Dentre as vantagens pode-se observar que são sistemas bem controlados, mais fáceis e rápidos para realização de testes em período curto, apresentam homogeneidade e possibilitam a utilização de drogas de maneira combinada com menor quantidade de fatores (como encontrada em amostras clínicas) que confundam a análise (NIU; WANG, 2015). Contudo, as principais limitações são: seleção fenotípica e genotípica das células durante a adaptação nas novas condições, isolamento das células do microambiente tumoral, mutações durante o decorrer

do tempo e isolamento das células do microambiente tumoral (CEKANOVA; RATHORE, 2014).

Portanto, a utilização das células *in vitro* permite que haja a avaliação do comportamento dessas células, com o desenvolvimento de ensaios sobre migração celular e realização de testes sobre estimuladores e inibidores de invasão celular, auxiliando na descoberta de novas drogas e suas respostas (BENTON et al., 2009).

3. RECEPTOR TIROSINA QUINASE

As proteínas quinases catalisam uma série de eventos na fosforilação e transferem um grupo fosforila (PO_3^{-2}) para proteínas alvo através de adenosina trifosfato (ATP). As proteínas tirosinas quinases são assim denominadas por apresentarem uma cadeia lateral fenólica e em humanos contém 90 membros dentro desta família de proteínas (ROSKOSKI, 2015). Dessas 90 proteínas, 58 são receptores tirosina quinase transmembrânicas e 32 são tirosina quinase não receptoras citoplasmáticas (GOCEK; MOULAS; STUDZINSKI, 2014; GSCHWIND; FISCHER; ULLRICH, 2004; ROSKOSKI, 2015).

Os receptores de tirosina quinase possuem um domínio extracelular de ligação, uma hélice transmembrana e um domínio intracelular catalítico (KRAUSE; VAN ETTEN, 2005). A maioria dos receptores tirosina quinase são monômeros na membrana celular, e a ligação de um fator de crescimento na região extracelular do receptor induz a dimerização desses receptores resultando em auto fosforilação (dependente de ATP) do domínio intracelular e ativação das cascatas de sinalização das quinases (GLÜCK et al., 2015; HUBBARD; MILLER, 2007; KRAUSE; VAN ETTEN, 2005; SUN; BERNARDS, 2014)(LEMMON; SCHLESSINGER, 2010).

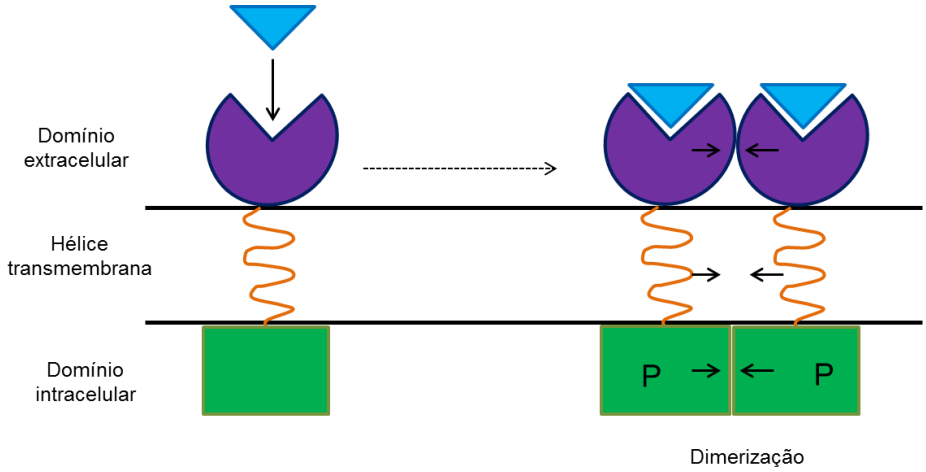

Figura 1: Estrutura de um receptor tirosina quinase monodímero (esquerda). Dimerização após ligação com fator de crescimento e posterior autofosforilação (P). Adaptado de (SCHLESSINGER, 2014).

Os receptores são essenciais para a transdução de sinal extracelular para o interior da célula e possuem papel de enzimas com atividade quinase (GOTINK; VERHEUL, 2010; PAUL; MUKHOPADHYAY, 2004). Participam em processos celulares fundamentais, como proliferação, diferenciação, migração, metabolismo, sobrevivência e comunicação celular durante desenvolvimento normal das células e tecidos (GSCHWIND; FISCHER; ULLRICH, 2004). Porém, mudanças ou anormalidades genéticas que alterem a atividade, distribuição celular, quantidade ou regulação dos receptores tirosina quinase, além de mutações e ativações aberrantes da sinalização intracelular estão ligados ao desenvolvimento do câncer, inflamação, doenças ósseas e angiogênese(LEMMON; SCHLESSINGER, 2010).

Os mecanismos associados à ativação patológica desses receptores são diversos, incluem mudanças estruturais (mutações pontuais ou proteína truncada, por exemplo), amplificação do gene e consequentemente aumento da expressão proteica, ativação excessiva através do ligantes e falta de mecanismos reguladores (TEMPLETON et al., 2014). Em cães, a desregulação dos receptores tirosina quinase em neoplasias é menos bem caracterizada, contudo as neoplasias com alterações nestes receptores têm apresentado mecanismos similares quando comparados ao humano (LONDON et al., 2003).

Nota-se que a participação dos receptores tirosina quinase tem sido relatada em diversas neoplasias em cães e humanos, portanto são alvos de drogas em diferentes tumores e podem representar uma opção em pacientes com metástase prostática (GALLICK et al., 2012; LONDON et al., 2003; OJEMUYIWA; MADAN; DAHUT, 2014). Em humanos com carcinoma prostático de maior risco ou que apresentem resistência à radioterapia, as drogas inibidoras de receptores de tirosina quinase estão entre os métodos alternativos (BROOKS et al., 2012; HOFER et al., 2004). Também é citada sua utilização e eficácia em outras neoplasias em humanos, como por exemplo, o uso de mesilato de imatinibe (Gleevec; Novartis, East Hanover, NJ, EUA), utilizado em casos de leucemia mieloide crônica e tumor gastrointestinal (GIST) (MOCELLIN et al., 2018; NISHIDA; DOI; NAITO, 2014). Além do mesilato de imatinibe, existem outras moléculas disponíveis para o tratamento do câncer, com diferentes proteínas quinase alvo, como mostra no quadro 1.

Nome	**Proteína quinase alvo**
Herceptin ™ (transtuzumabe)	HER2
Dacomitinib	HER1(EGFR), HER2, HER4
Iressa TM (ZD1839)	EGFR
SU6668	PDGFR/VEGFR
ZD6474	VEGFR/EGF
Sunitinibe	VEGF
Imatinibe	KIT e VEGFR-A

Quadro 1: Diferentes inibidores tirosina quinase para tratamento de neoplasias em humano e seus respectivos alvos (JE; SCHUTZ; CHOUEIRI, 2009; MANLEY et al., 2002; MOCELLIN et al., 2018)

Na medicina veterinária, os inibidores de tirosina quinase têm sido utilizados, dentre eles Palladia (toceranib), Kinavet (masitinib) e Gleevec (imatinib) em cães, e o Gleevec em gatos (LONDON, 2009). Foi aprovado o uso de fosfato de toceranib (Palladia®) em mastocitomas canino devido sua eficácia, porém também têm sido abordadas suas atividades contra outros tipos de neoplasias, incluindo alguns carcinomas (BERNABE et al., 2013). Em um

estudo com cães, foram observados nos carcinomas uma resposta parcial (carcinoma mamário metastático) ou uma evolução estável (carcinoma de células transicionais da bexiga, carcinoma pulmonar e carcinoma de células escamosas) quando administrados o Palladia® via oral (LONDON, 2009).

O fosfato de toceranib (Palladia®) possui semelhanças com outros inibidores disponíveis para pacientes humanos, como o imatinibe, sorafenibe e sunitinibe (KHANNA; GORDON, 2009). Esta molécula age como inibidor seletivo de múltiplos receptores tirosina quinase, entre eles VEGFR, PDGFR, c-KIT, e compete diretamente com a adenosina trifosfato (ATP), prevenindo a fosforilação do receptor e transdução do sinal (YU et al., 2014). De acordo com observações em modelos experimentais de laboratório, a ativação de uma única proteína quando comparada com ativação de múltiplas proteínas quinases apresenta um fenótipo tumoral menos agressivo, portanto sugere-se que a inibição de diversos receptores tirosina quinase, como o Palladia®, produzirá um efeito antitumoral mais efetivo do que uma inibição única (STOMMEL et al., 2007).

3.1 Receptor de Fator de Crescimento do Endotélio Vascular (VEGFR)

As células tumorais e metastáticas, assim como as células normais, necessitam de nutrientes, oxigênio, fatores de crescimento, enzimas, hormônios, entre outras substâncias, além de habilidade de eliminar metabólitos e dióxido de carbono (CO2)(HANAHAN; WEINBERG, 2011; HICKLIN; ELLIS, 2005). Portanto, para a sobrevivência e proliferação dessas células é necessário que haja a angiogênese, o qual é o processo de formação de novos vasos sanguíneos através de redes vasculares pré-existentes. Durante esse processo as células endoteliais se dividem e são incorporadas nos novos capilares (ROSKOSKI, 2007).

A angiogênese é regulada por diversos fatores de crescimento e citocinas que estão presentes em condições normais, entretanto os níveis desses fatores se equilibram após a perfusão sanguínea ter sido estabelecida (MOENS et al., 2014). Dentre esses fatores está o fator de crescimento vascular endotelial (VEGF) que se liga a seus receptores (VEGFR1, VEGFR2, VEGR3), sendo o VEGFR2 um importante fator em situações patológicas que

envolvem a neovascularização tumoral (GOTINK; VERHEUL, 2010; YU et al., 2014).

Em condições patológicas há geração de vasos sanguíneos com anormalidades estruturais e funcionais, pois os sinais pró angiogênicos geralmente excedem os sinais anti angiogênicos e fatores de maturação do vaso (WANG et al., 2015). Dentre os efeitos, observa-se a falta de conexão das células endoteliais e pericitos, o que compromete a função da barreira endotelial vascular, permitindo passagem do plasma e células, inclusive tumorais (BALUK; HASHIZUME; MCDONALD, 2005; MORIKAWA et al., 2002). A passagem do plasma faz com que a pressão intersticial aumente e impede a distribuição de droga, também altera o transporte adequado de nutrientes e oxigênio, e consequentemente as células neoplásicas estimulam a angiogênese em um ciclo compensatório (POTENTE; GERHARDT; CARMELIET, 2011).

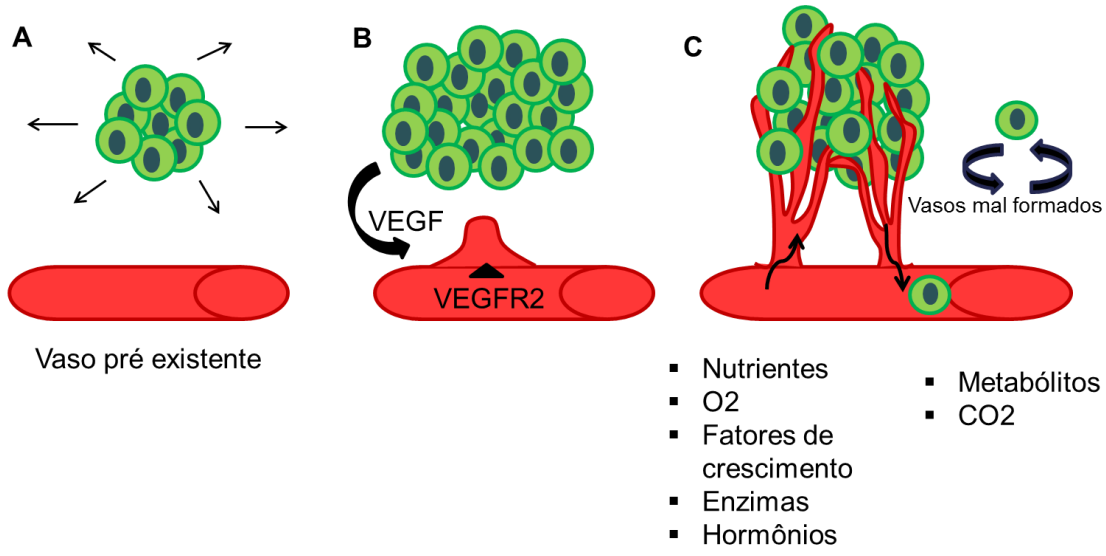

Figura 2: Papel da angiogênese na carcinogênese. **A)** Quando o tumor é pequeno, as células neoplásicas se mantêm com o oxigênio e nutrientes de vasos pré-existentes. **B)** Conforme as células neoplásicas se multiplicam, há liberação de sinais pró angiogênicos, como por exemplo, o VEGF que se liga ao VEGFR2 para que haja proliferação de novos vasos. **C)** Os novos vasos que são mal formados permitem a nutrição das células, mas também permitem a passagem do plasma e células para o interstício, impedindo transporte adequado de oxigênio e nutrientes. Portanto as células estimulam ainda mais a angiogênese, em um ciclo compensatório.

Imagem: Adaptado Vasudev & Reynolds (2014)

O VEGF representa um dos agentes promotores angiogênicos tumorais mais potentes e permite aumento da atividade angiogênica, mitogênica e

permeabilidade vascular específica para as células endoteliais, com função importante na formação de metástases distantes (WERGIN; KASER-HOTZ, 2004). Em câncer prostático humano, os níveis de VEGF no sangue e urina parecem estar correlacionados com o resultado dos pacientes e a densidade microvascular tem sido utilizada para predizer a progressão tumoral e relacionada com grau mais elevado no escore de Gleason (HWANG; HEATH, 2010). A densidade microvascular nos carcinomas é mais alta quando comparada às próstatas normais, e os carcinomas por sua vez apresentam maior densidade microvascular quando possuíam metástases, sugerindo uma relação entre densidade microvascular e estágio tumoral (ARYA et al., 2006).

Diante da importância da angiogênese no crescimento tumoral e metástases, a utilização de inibidores incluindo VEGFR e seu ligante (VEGF) tem sido uma estratégia eficaz em alguns tipos tumorais associados a quimioterapias, porém no carcinoma prostático não está bem definido e ainda apresenta uma área promissora de pesquisa (BILUSIC; WONG, 2014; COHEN et al., 2007; MUKHERJI et al., 2013; RAJABI; MOUSA, 2017; WANG; JAIN; BATCHELOR, 2017)

3.2 Receptor do Fator de Crescimento Derivado de Plaquetas (PDGFR)

Existem no total quatro genes de fator de crescimento de plaquetas (PDGF A, B, C e D) que produzem cinco tipos de proteínas biologicamente ativas: quatro homodímeros (PDGF AA, BB, CC, DD) e uma heterodímera (PDGF AB) (NAJY et al., 2012). Há dois receptores tirosina quinase: PDGFRα e PDGFRβ, o primeiro se liga a todas as isoformas da proteína, exceto PDGF DD, enquanto que o segundo receptor é ativado pela isoforma PDGF BB e PDGF DD (DEMOULIN; ESSAGHIR, 2014). Ambos os receptores podem ser ativados diretamente pela ligação da proteína VEGF-A (PENNOCK; KAZLAUSKAS, 2012).

O PDGF é secretado por plaquetas ativadas e diversas outras células: endotelial, epitelial, glial e células inflamatórias (DEMOULIN; ESSAGHIR, 2014). Em condições fisiológicas, as ações biológicas das PDGFs e seus receptores (PDGFRs) são controladas de maneira parácrina, reguladas pelo microambiente celular, citocinas e estímulos de crescimento, e participam em processos de cicatrização, inflamação e angiogênese (CAO, 2013; RANIERI et

al., 2013). A sinalização via PDGFRα atua no desenvolvimento de folículo piloso, oligodendrócitos, pulmão e vilosidade intestinal, enquanto que a via PDGFRβ é importante no desenvolvimento de vasos sanguíneos, rins e adipócito unilocular (HELDIN, 2013; KARLSSON et al., 2000; SORIANO, 1997).

Entretanto, na tumorigênese, os membros da família PDGF interagem com os receptores tirosina quinase localizados na transmembrana e implica em sinalização através de diversos mecanismos: sinalização autócrina proliferativa, angiogênesis e contribuição na transição epitelial mesenquimal, contribuindo no comportamento invasivo e metastático da neoplasia (HWANG; HEATH, 2010). Existem ainda estudos que comprovam a ativação do PDGFR através de mecanismos que não sejam a ligação direta das proteínas PDGF (RUSSELL et al., 2010).

PDGFs e PDGFRs também possuem papel importante no estroma tumoral através do recrutamento de pericitos e células musculares lisa do vaso, que por sua vez estabilizam os vasos neoformados e estimulam as células do estroma a produzir VEGF-A e consequente angiogênese (KILVAER et al., 2014). Em diferentes tipos de neoplasias, o PDGFRβ também é expresso vinculado a fibroblastos associados ao câncer, demonstrando que o PDGFRs estromais podem regular múltiplas etapas da carcinogênese, metástase e interferir na eficácia da droga (ÖSTMAN, 2017). Em um estudo realizado em ratos, utilizado com modelo de câncer prostático, mostrou que a terapia alvo de PDGFR-β estromal resultou em densidade vascular diminuída, aumento de apoptose celular e menor crescimento tumoral quando comparados com grupos que realizaram apenas a castração como terapia (NIU; XIA, 2009).

Em humanos, nos carcinomas prostáticos e suas metástases ósseas o PDGFR-β geralmente está aumentado em 88% e 80% dos casos, respectivamente (NAJY et al., 2012), além da expressão proteica de PDGFR em lesões pré neoplásicas (neoplasia intraepitelial), sugerindo um papel deste receptor na progressão tumoral (HOFER et al., 2004). Em linhagens celulares de carcinoma prostático celulares (PC3) foi demonstrado um aumento de PDGF-D, com consequente transição epitelial mesenquimal e um fenótipo de célula tronco, o que pode explicar o aumento da invasividade (KONG et al., 2010).

Diferentes estratégias têm sido desenvolvidas na utilização de terapias inibidoras em doenças que o PDGFR possui um papel importante, com resultados positivos em associação com outros tratamentos, como em casos de glioblastoma (REARDON et al., 2005; ROBERTS; COSSIGNY; QUAN, 2013). Entretanto, os estudos com inibidor de receptor tirosina quinase nos carcinomas prostáticos, assim como em diferentes neoplasias, ainda apresentam muitos efeitos colaterais e ineficácia, necessitando de maiores estudos (HELDIN, 2013).

3.3 c-KIT

O proto oncogene *c-KIT* codifica um receptor de tirosina quinase de transmembrana, denominado c-Kit (também nomeado CD117), e estruturalmente está relacionado com outras receptores transmembrânicos: VEGFR e PGDFR (ARBER; TAMAYO; WEISS, 1998; QIU et al., 1988). O receptor c-KIT tem como ligante a proteína SCF (fator de célula tronco), fator de crescimento que existe como glicoproteína ligada a membrana e também na isoforma solúvel (LIANG et al., 2013; ROSKOSKI, 2005).

O sistema SCF/c-KIT é um sistema expresso em diversas células e tecidos: fibroblastos, células endoteliais, células tronco hematopoiéticas, germinativas, prostáticas, endometriais, intersticiais de Cajal, mastócitos, melanócitos, entre outros (FIGUEIRA et al., 2015; LAMMIE et al., 1994; LENNARTSSON; RONNSTRAND, 2012; ROSKOSKI, 2005). A interação de ambos possuem papel importante nas vias de ativação da diferenciação, proliferação, apoptose e sobrevivência celular (DI LORENZO et al., 2004; EDLING; HALLBERG, 2007). A proteína SCF é uma citocina inflamatória e considerada a maior ativadora de mastócitos, os quais levam a uma mudança no microambiente tumoral devido ao aumento de inflamação e imunossupressão (HUANG et al., 2008).

Entretanto, uma correlação positiva entre desregulação do *c-KIT* e a transformação maligna de diferentes células tem sido descrita (PARONETTO et al., 2004; SHI et al., 2016). Uma das alterações é devido a mutações que ativam o receptor tirosina quinase independente de ligantes ou que codificam o c-KIT truncado independente de ATP para ativá-lo (ABBASPOUR BABAEI et al., 2016; TOYOTA et al., 1994). Em humanos as mutações em éxons

específicos são reportadas em diversos tumores, como é o caso de tumores gastrointestinais (GISTs), leucemia mielóide e seminomas testiculares (BLUME-JENSEN; HUNTER, 2001; HEINRICH et al., 2002). Em mastocitomas caninos também é relatada mutações do proto oncogene *c-KIT* com alta graduação histológica (ZEMKE; YAMINI; YUZBASIYAN-GURKAN, 2002). .

Além disso, durante a carcinogênese é relatado o aumento na expressão do receptor tirosina quinase, sensibilidade aumentada em relação ao seu ligante SCF, e estímulo autócrino (células tumorais) ou parácrino (microambiente tumoral) (ABBASPOUR BABAEI et al., 2016; PARONETTO et al., 2004). Carcinomas prostáticos humanos apresentam aumento da expressão proteica de SCF quando comparados com tecido prostático normal, além de correlação positiva com os carcinomas mais agressivos (ALABIAD et al., 2018). Em relação ao receptor c-KIT, observou-se maior expressão proteica em lesões metastáticas do que em tumores primários de carcinoma prostático humano, sugerindo uma interação entre células neoplásicas e microambiente tumoral ósseo na progressão da doença (MAINETTI et al., 2015). Em carcinomas prostáticos caninos a expressão proteica de c-KIT apresenta heterogeneidade de marcação e possivelmente sem relação com a metástase, porém todos os CaP classificados com escore de Gleason 10 apresentavam a proteína, sugerindo uma possibilidade de tratamento personalizado com inibidores de c-KIT nestes casos (FONSECA-ALVES et al., 2017).

4. INIBIDOR DE mTORC (RAPAMICINA)

A rapamicina é um macrolídeo descoberta como um metabólito produzido por *Streptomyces hygroscopicus* e inicialmente utilizada como antifúngico (VÉZINA; KUDELSKI; SEHGAL, 1975). Posteriormente, o fármaco passou a ser utilizado como terapia imunossupressora após transplante para minimizar a rejeição do órgão, pois inibe a habilidade das células dendríticas sofrerem maturação em células apresentadora de antígeno (APC), as quais estimulam as células T (BAROJA-MAZO et al., 2016). Além disso, possui funções anti proliferativas em células de mamíferos, pois inibe a proteína mTOR (proteína alvo da rapamicina em mamíferos), a qual seu nome é derivado devido a ação do fármaco (XIE; WANG; PROUD, 2016).

A proteína mTOR é uma proteína serina/treonina quinase, e é classificada desta forma pois contém uma cadeia lateral alcóolica (ROSKOSKI, 2015). Estruturalmente (figura 3) é composta pela porção N-terminal que possui duas repetições HEAT (fator de alongamento da huntingtina3, subunidade da proteína fosfatase 2A e a quinase Tor 1) em tandem (ZHOU; HUANG, 2010). A extremidade C-terminal da proteína possui o domínio FAT e FATC, ambos necessários à função catalítica (YANG et al., 2013). Adjacente ao domínio FAT, há o domínio ligante FKBP12-rapamicina (FRB), que se liga ao complexo mTOR e FKBP, os quais estão ligados a rapamicina (NASR et al., 2015). O domínio quinase catalítico (KIN) possui uma pequena região chamada de domínio regulatório negativo (NRD) que possui sítios de fosforilação da treonina 2446, serina 2448 e serina 2481, e relacionadas com maior atividade de mTOR quando fosforiladas (HOEFFER; KLANN, 2010).

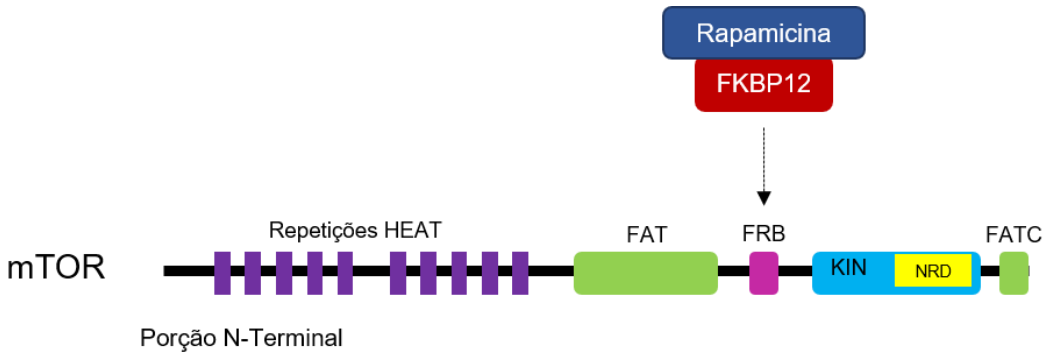

Figura 3: Representação estrutural esquemática da proteína mTOR. Porção N-terminal com duas repetições HEAT em tandem. Domínio FAT e FATC apresentam função catalítica, seguido de domínio FRB, o qual se liga ao complexo FKBP12-rapamicina, e, portanto, onde atua o fármaco (rapamicina). O domínio quinase catalítico (KIN) possui um domínio repressor (NRD). Adaptado (LI et al., 2014).

A proteína mTOR está presente em duas formas distintas de complexo multiproteico, denominados complexos 1 e 2 da mTOR, mTORC1 e mTORC2, respectivamente (BALLOU; LIN, 2008; MOCELLIN et al., 2018). O primeiro complexo citado é classificado como sensível e o segundo insensível a rapamicina (BALLOU; LIN, 2008; LI et al., 2016). Em ambos os complexos, a proteína mTOR é a subunidade catalítica (DAN et al., 2014). Cada um desses complexos apresenta proteínas diferentes em sua composição, além de fosforilar diferentes substratos (KENNEDY; LAMMING, 2016).

O mTORC1 consiste nas proteínas mTOR, mLST8 (proteína 8 Sec13 mamífero letal), Raptor (proteína regulatória de mTOR), e dois inibidores endógenos do complexo: DEPTOR (proteína de interação mTOR contendo domínio DEP) e PRAS 40 (substrato de AKT rico em prolina)(DIBBLE; CANTLEY, 2015). Após ativação de mTORC1, o complexo fosforila diretamente as proteínas PRAS 40 e DEPTOR, reduzindo interação com o complexo e ativando a sinalização deste complexo (LAPLANTE; SABATINI, 2009). O complexo 2 da mTOR contém as proteínas mTOR, mLST8, Rictor (proteína associada a mTOR insensível a rapamicina) e mSIN1 (proteína de interação da quinase de mamíferos ativada por estresse 1) (DAN et al., 2014).

mTORC1 é sensível a rapamicina, pois a interação do fármaco com a proteína FKBP12 (proteína de ligação FK506), resulta em um complexo que se liga ao domínio FRB da proteína mTOR (HAUSCH et al., 2013; MENG; ZHENG, 2015). Consequentemente, interrompe a ligação com a proteína Raptor , bloqueando assim a sinalização da via PI3K/AKT/mTOR (BOVÉ; MARTÍNEZ-VICENTE; VILA, 2011). O mTORC1 é responsivo a fatores nutricionais celulares e do organismo, incluindo aminoácidos, glucose e oxigênio (DAN et al., 2014; DIBBLE; MANNING, 2013; RAMIREZ-RANGEL et al., 2011). Comparado ao mTORC1, as vias que regulam mTORC2 são muito menos conhecidas, sendo a via PI3K a única via descrita (OH; JACINTO, 2011). Além disso, devido à ausência de ligação do complexo FKP12-rapamicina ao mTORC2, sugere-se inibição da rapamicina apenas a mTORC1. Porém, em algumas células, o uso prolongado de rapamicina (mais de 24 horas) tem apresentado supressão de mTORC2, contribuindo no efeitos do medicamento (SARBASSOV et al., 2006).

O complexo 1 controla diferentes vias, incluindo a síntese e produção de ribossomos, transcrição, apoptose, lipogênese e síntese de nucleotídeo, os quais são funções importantes para crescimento celular e tecidual (XIE; WANG; PROUD, 2016). Este complexo é mediado por substratos que incluem o fator de iniciação eucariótico 4E (4E-BP1), proteína quinase ribossomal 6S (S6K1), ULK, entre outros (KENNEDY; LAMMING, 2016). Também participa da via fosfatidilinositol-3 -quinase (PI3K) /AKT/mTOR, a qual regula o crescimento e sobrevivência celular por meio do controle da taxa de processo anabólico, capacidade de síntese proteica e atividade mitocondrial (KIM; GUAN, 2015).

A desregulação da via PI3K/AKT/mTOR principalmente devido hiperativação de mTOR está descrita em diferentes tipos de câncer, incluindo em câncer prostático (HSIEH et al., 2015) (MABUCHI et al., 2015). A perda de função de genes supressores, incluindo PTEN (homólogo da fosfatase e tensina) e TSC 1 / 2 (complexo esclerose tuberosa), ou a mutação em oncogenes , por exemplo KRAS, AKT, PIK3CA, também estão relacionadas com a hiperativação da via PI3K/AKT mTOR (JHANWAR-UNIYAL et al., 2019).

A ativação da via PI3K/AKT/mTOR se dá pela fosforilação da classe IA PI3Ks na membrana celular (figura 3), através de sinais extracelulares, como por exemplo insulina, ativação do receptor de fator de crescimento epidérmico (EGFR), oncogenes , dentre outros (KIM; GUAN, 2015; LOPICCOLO et al., 2008; MABUCHI et al., 2015). A classe I da proteína PI3K possui uma subunidade catalítica (p110) e reguladora (p85), e após fosforilação da PI3K a subunidade p110 fosforila o componente lipídico da membrana celular: fosfatidilinositol-3,4 bifosfato (PIP2) em fosfatidilinositol-3,4,5 bifosfato (PIP3) (LI et al., 2016). Posteriormente, a proteína AKT é fosforilada e ativa diferentes proteínas, incluindo o complexo 1 da mTOR que por sua vez regula outros substratos, como por exemplo, fosforila a proteína 1 ligante (4E-BP1) do fator de iniciação eucariótico 4E (elF-4E) e S6Ks, os quais regulam a síntese proteica (KIM; GUAN, 2015; ZHANG et al., 2017). A proteína 4E-BP1 liga-se a elF-4E, porém após sua fosforilação esta associação entre ambas é desfeita, permitindo a tradução proteica (QIN; JIANG; ZHANG, 2016).

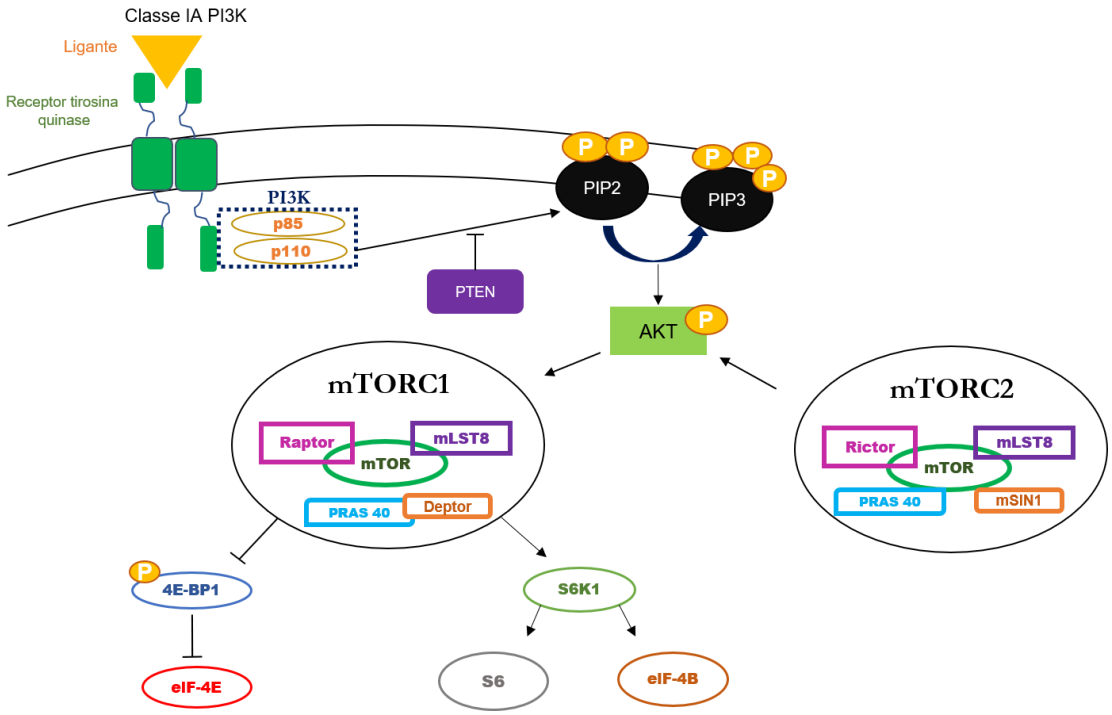

Figura 4: Via PI3K/AKT/mTOR simplificada. Ativação da classe IA PI3K com as subunidades p85 e p110, subsequente fosforilação (P) de PIP2 em PIP3, posterior fosforilação de AKT e ativação do complexo 1 mTOR (mTORC1). Ativação de mTORC1 resulta em ativação de S6K1, e fosforilação da proteína 4E-BP1 gera diminuição de afinidade com a proteína eIF-4E, soltando-a. Ativação de mTORC2 resulta em fosforilação de AKT.

Em cães, o aumento de proteínas relacionados com a ativação da via PI3K/AKT/mTOR têm sido descritos em diferentes neoplasias, como por exemplo hemangiossarcoma (MURAI et al., 2012b), carcinoma prostático (RIVERA-CALDERÓN et al., 2019b), células de melanoma (KENT; COLLINS; YE, 2009) e células de osteossarcoma (GORDON; YE; KENT, 2008b). Nos carcinomas prostáticos caninos com alto escore de Gleason (maior ou igual a oito) foi observado aumento de expressão proteica de mTOR fosforilado e eIF4E quando comparado a próstata normal (RIVERA-CALDERÓN et al., 2019b). Além disso, uma diminuição na expressão proteica de PTEN nos carcinomas prostáticos caninos foi reportada (RIVERA-CALDERÓN et al., 2016). Esses achados indicam alterações de proteínas envolvidas na ativação da via PI3K/AKT/mTOR e, portanto, sendo uma via de possíveis alvos terapêuticos no tratamento de carcinoma prostático em cães.

5. OBJETIVOS

5.1 Objetivos gerais

O presente estudo tem como objetivo geral avaliar o efeito do fosfato de toceranib (Palladia®) e rapamicina na viabilidade celular e o perfil transcricional das células epiteliais neoplásicas de cultura celular advindas de dois carcinomas prostáticos caninos distintos, referidas como PC1 e PC2.

5.2 Objetivos Específicos

- Avaliar a expressão gênica e proteica de VEGFR2, PDGFR e c-KIT nas células PC1 e PC2.
- Analisar a viabilidade celular das células PC1 e PC2 *in vitro* após tratamento com o fosfato de toceranib (Palladia®) e rapamicina, e identificar a concentração inibitória para 50% das células em ambos os tratamentos.
- Avaliar o perfil de expressão gênica entre as células PC1 e PC2 tratadas ou não com fosfato de toceranib.
- Avaliar o transcriptoma das células PC1 e PC2 com células epiteliais advindas de próstata normal.
- Avaliar a expressão gênica dos genes *AKT*, *mTOR* e *4E-BP1* antes e após tratamento com rapamicina das células PC1 e PC2.
- Avaliar a expressão proteica de AKT fosforilado (p-AKT), mTOR fosforilado (p- mTOR) e 4E-BP1 fosforilado (p- 4E-BP1) antes e após o tratamento com rapamicina das células prostáticas caninas (PC1 e PC2).

Capítulo 2- Trabalho científico 1

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Transcriptome of Canine Prostatic Carcinoma Cells Treated with Toceranib Phosphate Revels Distinct Antitumor Profiles Associated with the PDGFR Pathway

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Abstract

Canine prostate cancer (PC) presents a poor antitumor response and prognosis. Toceranib phosphate (TP) is a multiple tyrosine kinase (TK) receptor inhibitor, including VEGFR, PDGFR and c-KIT. This study aimed to evaluate VEGFR2, PDGFR-β and c-KIT protein expression in two canine PC cell lines (PC1 and PC2) and to evaluate the transcriptome profile of the cells after TP treatment. Immunofluorescence analysis revealed VEGFR2 and PDGFR-β protein expression and the absence of c-KIT protein expression in both cell lines. After TP treatment, only PC1 cell viability decreased in a dose-dependent manner. Transcriptome and enrichment analyses of treated PC1 cells revealed 181 upregulated, which were related to decreased angiogenesis and cell proliferation. In addition, we found upregulated *PDGFR-A*, *PDGFR-β*, *PDGF-D* expression and can represent a potential mechanism of resistance. The upregulation of *PDGFR-β* was also observed in treated PC1 cells by qPCR. PC2 cells had fewer protein-protein interactions, with 18 upregulated and 22 downregulated genes. The upregulated were involved in the regulation of parallel pathways and mechanisms related to proliferation that could be connected to the resistance observed after treatment. We suggest that due to heterogeneity neoplastic cells, cancer therapy should be personalized and that toceranib phosphate may have biological activity in canine PC, but resistance still remains a particular concern.

Key words: dog; prostate; microarray; antitumor response; animal model.

1. Introduction

Although the prevalence of prostate cancer (PC) in dogs is relatively low, other than humans, canines are the only domestic species known to spontaneously develop PC [1,2]. Canine prostate carcinoma is a biologically aggressive neoplasm that exhibits a poor prognosis related to its late diagnosis, high metastatic rate (80% at death) and limited effective treatments [1,3,4]. Some reported metastatic sites are the lungs, bone, lymph nodes, liver, spleen and colon [4–8].

Different from humans, PC in dogs is not androgen dependent, so androgen deprivation therapy is not efficient [9]. Due to some important differences between PCs in men and dogs, many treatment modalities used successfully in human medicine fail to be applicable in dogs [9]. Therefore, there is a need for new therapies for canine PC, including targeted therapies, which are drugs that target specific proteins in neoplastic cells or tumor-associated antigens, with less damage to normal cells [10].

Toceranib phosphate, the veterinary counterpart to sunitinib (SU11248), works by preventing receptor tyrosine kinase (RTK) phosphorylation and downstream signaling molecules (Liao, 2002; London et al., 2003; London, 2004). Toceranib phosphate is a multiple RTK inhibitor, with targets including VEGFR (vascular endothelial growth factor receptor), PDGFR (platelet-derived growth factor receptor) and c-KIT, that is approved for the treatment of dogs with cutaneous mast cell tumors and originally developed as an antiangiogenic agent [14–16]. Additionally, some reports of phase I clinical trials in dogs suggest that toceranib phosphate treatment exhibits clinical antitumor activity against different cancers, including gastrointestinal stromal tumors, lymphomas, multiple myelomas, metastatic soft tissue sarcomas, and several carcinomas, such as metastatic mammary, head and neck, thyroid and prostatic carcinomas [11,17–19].

Although some studies have demonstrated the clinical anticancer effect of toceranib in dogs, the mechanisms of action have not been studied. We used global gene expression analysis in two canine PC cell lines to evaluate the genes involved in the treatment response to toceranib phosphate. To investigate the molecular mechanism underlying toceranib phosphate

cytotoxicity in canine PC cell lines, we evaluated viability and gene expression alterations in response to toceranib phosphate.

2. Materials and methods

2.1 Ethical approval

This study was approved by the Ethics Committee on Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science of the São Paulo State University, Botucatu (Protocol: 0004/2017), regulated by normative resolution 12, from the Ministry of Science, Technology and Innovation. All procedures of this study were performed in accordance with the National and International Recommendations for the Care and Use of Animals (National Research Council)[20].

2.2 Primary canine PC cell culture

Two characterized primary canine prostate cancer cell cultures (PC1 and PC2) from two different canine PCs collected during necropsy were cultured as previously described in another study [21]. PC1 and PC2 cells were cultured in complete medium with DMEM F12 (Lonza Inc., Allendale, NJ, USA) at 37 °C in 5% $CO_{2;}$ the culture medium supplemented with 10% inactivated fetal bovine serum (FBS, HYCLONE, Waltham, MA, USA) and 100 U/mL of penicillin G and 100 mg/mL of streptomycin (SIGMA, Portland, OR, USA).

2.3 Protein expression by immunofluorescence (IF)

To evaluate TK receptors in these cell lines, we performed IF for VEGFR2, PDGFR-β and c-KIT. PC1 and PC2 cells were seeded at $5x10^4$ cells/well into 12-well chamber slides (SPL Life Sciences), allowed to adhere to the coverslips at 37°C and 5% CO_2, until reaching approximately 70% confluence. Cells were then washed using Dulbecco´s phosphate-buffered saline (DPBS) at 4ºC and fixed in methanol for 30 minutes at 27ºC. Samples were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at 27ºC and incubated with commercial reagent (Protein Block Serum Free, Dako®) for 30 minutes to block nonspecific antibody binding. The cells were incubated for 18 hours at 4°C in a humidified atmosphere with primary antibodies against VEGFR2 (Clone SC-6251, Santa Cruz Biotechnology), PDGFR-β (Clone 3162, Cell Signaling

Technology®) and c-KIT (Clone A4502, Dako®), all of which were diluted 1:100. Next, the cells were incubated with the following secondary antibodies at 1:10,000 dilutions: Alexa Fluor 594 (Clone Poly4053, BioLegend) for VEGFR, Alexa Fluor® 488 (Clone A11034, ThermoFisher Scientific) for PDGFR-β and c-KIT (CD117). The samples were then labeled with DAPI (4,6-diamidino-2-phenylindole dihydrochloride) (D9542, Dako®) at a 1:10,000 dilution and examined and imaged using a TCS SP5 confocal microscope (Leica biosystems, Wetzlar, Alemanha).

The fluorescent results were considered positive or negative according to presence or absence of protein.

2.4 MTT assay and IC50 detection

PC1 and PC2 cells were seeded into 96-well plates at a concentration of $1x10^4$ cells/well in 0.1 ml of complete and allowed to grow for 24 hours in a 5% CO_2 incubator. The complete medium was removed and incubated with different concentrations of toceranib phosphate (Sigma-Aldrich): 3 µM, 6 µM, 9 µM, and 12 µM (12 wells per concentration); the drug was diluted in complete medium without inactivated fetal bovine serum and added for 24, 48 and 72 hours. There were two control groups: one with no treatment and the other with 0.4% DMSO as the vehicle control, which had no influence on the viability of the cells, compared with cells with no treatment.

MTT solution was prepared with 0.013 g of MTT (Invitrogen™, M6494) dissolved in PBS (2.5 ml), and 1 ml of MTT was diluted in 10 ml of serum-free medium to a final concentration of 0.5 mg/ml. Samples were incubated at 37ºC for 4 hours, and finally, the medium was removed from all plates. Precipitated MTT salts were solubilized by adding 200 µL dimethyl sulfoxide (DMSO - Hybri-Max™; Sigma) and incubated for 15 minutes. Absorbance values were determined at 595 nm on a multiwall plate reader.

Percentage of cell viability was calculated using the following formula: $(A_{treatment} - A_{blank})/ (A_{DMSO} - A_{blank}) \times 100\%$ [22].

Considering A = absorbance, DMSO = vehicle control, blank = no cells.

The IC50 calculation was performed using GraphPad Prism 8.0.

2.5 Quantitative PCR

After establishing the toceranib IC_{50} for each cell line, we treated PC1 and PC2 cells with the toceranib IC_{50} dosage (treated cells) for 24 hours and extracted RNA for RT-qPCR and transcriptome analysis.

This assay was performed in duplicate, and as a control, DMSO was added to nontreated cells. Total RNA isolation and purification were performed with a commercial kit, according to the manufacturer´s instructions (RNeasy mini kit, Qiagen, Hilden, Germany). The RNA concentration and purity were evaluated by spectrophotometry (NanoDrop™, ND-8000, Thermo Scientific, Waltham, MA, USA). RNA integrity was accessed by the Bioanalyzer 2100 and the Agilent RNA 6000 Nano Series kit, according to the manufacturer´s instructions (Agilent Technologies, Santa Clara, CA, USA).

cDNA synthesis was carried out using 1 µg of total RNA treated with DNAse I (Life Technologies, Rockville, MD, USA), 200 U of *SuperScript* III Reverse Transcriptase enzyme (Life Technologies), 4 µL of SuperScript First-Strand Buffer 5X, 1 µL of dNTP 10 mM each (Life Technologies), 1 µL of Oligo-$(dT)_{18}$ (500 ng/ µL) (Life Technologies), 1 µL of random hexamers (100 ng/ µL) (Life Technologies), and 1 µL of DTT 0.1 M (Life Technologies). Reverse transcription was performed at 50ºC for 60 min and inactivated at 70ºC for 15 min. qPCR amplification for *VEGFR2, PDGFR-β* and *KIT* reference genes (*GAPH, HPRT*, *RPS19*, *RPS5* and *RPL8)* was performed using QuantStudio 12k Flex Thermal Cycler equipment (Applied Biosystems; Foster City, CA, USA). The reactions were performed in duplicate in 384-well plates, using Power SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA, USA), 1µL of cDNA and 0.3 µM of each primer. Relative gene quantification was calculated by the $2^{-\Delta\Delta CT}$ method [23].

2.6 Microarray

We generated a global gene expression profile (microarray) using GeneChip® Gene 1.0 ST Arrays (Affymetrix, CA, EUA). Labeled cDNA, hybridization and detection were performed according to the manufacturer´s instructions. Then, the chips were scanned in the Scanner 3000 7G series (Affymetrix, Santa Clara, CA, EUA). Affymetrix CEL files were downloaded and processed by the Applied Biosystem™ Transcriptome Analysis Console (TAC,

Affymetrix) Software. The criteria for selecting differentially expressed genes were a 2.0-fold change cutoff and a P value <0.05. Hierarchical clustering heatmaps and Venn diagrams were generated using the TAC software.

2.7 Gene ontology

The differentially expressed genes between the groups were subjected to a gene ontology (GO) enrichment analysis using Enrichr (https://amp.pharm.mssm.edu/Enrichr/). Revigo (http://revigo.irb.hr/) was used to organize and visualize the enriched GO terms obtained from Enrichr. GO analysis was focused on two major categories: biological process and molecular function.

2.8 Protein-protein interactions (PPI) networks

The upregulated and downregulated DEGs were submitted independently to the online Search Tool for the Retrieval of Interacting Genes - STRING (https://string-db.org/) to generate PPI networks. We considered only STRING interactions of high confidence (0.700), and active interactions were databases, coexpression, neighborhood and cooccurrence. To simplify the network, we hid the disconnected nodes.

2.9 Statistical analysis

Comparisons between the different doses in the treatment groups were made using the Tukey-Kramer test, and statistical significance was set at $p<0.05$. *S*tatistical analysis was performed, and graphs were generated using Graph Pad Prism 8.0 and Microsoft ® Excel 2007.

3. Results

3.1 Protein expression by immunofluorescence

PC1 and PC2 samples showed cytoplasmic expression of VEGFR2 and PDGFR-β proteins. However, c-KIT protein was absent in both samples (figure 1).

3.2 MTT assay and IC50 detection

Toceranib phosphate inhibited PC1 cell viability in a dose dependent manner. After 24 hours, toceranib phosphate reduced cell viability to 91, 84, 59 and 0% in PC1 cells at concentrations of 3 µM, 6 µM, 9 µM and 12 µM, respectively; at the same concentrations, after 48 hours, cell viability was reduced to 93, 80, 50 and 2%, and after 72 hours, cell viability was reduced to 89, 73, 54 and 3%(figure 2).

Following treatment with toceranib phosphate for 24 (figure 2A), 48 (figure 2B) and 72 hours (figure 2C), a significant ($p<0.05$) decrease in the number of viable cells was observed in the PC1 cells when compared to that of the control cells (DMSO). The IC_{50} values of toceranib phosphate were 9.28 µM, 9.13 µM and 8.95 µM for PC1 cells at 24 (figure 2D), 48 (figure 2E) and 72 (figure 2F) hours, respectively.

On the other hand, toceranib phosphate did not reduce PC2 cell viability in a dose dependent manner. After 24 hours (figure 2G), the treatment reduced cell viability to 82, 90, 84 and 84% compared to that of the control at concentrations of 3 µM, 6 µM, 9 µM and 12 µM, respectively; at the same concentrations, after 48 hours (figure 2H), cell viability was reduced to 70, 85, 72 and 65%, and after 72 hours (figure 2I), cell viability was reduced to 86, 73, 78 and 74%. Thus, we considered the PC2 cell line to be more resistant to toceranib than the PC1 cell line.

3.3 qPCR

VEGFR2 transcript levels were statistically ($p<0.05$) lower in treated PC1 (mean=1.017) cells in comparison with untreated samples (mean = 0.849) (figure 3A), however no significant differences were observed in the *VEGFR2* gene expression among the PC2 untreated and treated with toceranib phosphate (figure 3B). There was a significant upregulation of *PDGFR-β* in PC1 (figure 3C) comparing untreated (mean = 1.007) and treated samples (mean= 2.056). We observed downregulation of *PDGFR-β* in PC2 (figure 3D) comparing untreated (mean= 1.001) and treated (0.549) cells. On the other hand, treatment with toceranib phosphate for 24 hours did not alter transcript *KIT* levels between untreated and treated cells (PC1 and PC2) (figure 3E and figure 3F, respectively).

3.4 Transcriptome analysis

A total of 390 differentially expressed genes (DEGs) ($p<0.05$) were observed comparing untreated and toceranib phosphate-treated PC1 cells, including 233 upregulated genes and 157 downregulated genes in treated cells. However, 122 DEGs were not annotated with gene names or gene symbols in TAC software, and 5 were microRNAs.

A total of 82 DEGs were observed ($p<0.05$) comparing PC2 untreated and treated cells, including 42 upregulated genes and 40 downregulated genes in treated cells. However, the DEG list showed that out of 82 genes, 42 genes had neither gene name nor symbol, and 1 gene was actually a microRNA. A heatmap of these DEGs is shown in figure 4A and DEGs were clustered which can well differentiate the treated from the untreated, and PC1 and PC2 cells.

Venn diagram (figure 4B) was used to compare the lists of DEGs between PC1 (treated and untreated) and PC2 (treated and untreated) cells. In a total of 525 DEGs, only 17 common DEG between the two comparisons were screened out. Among these genes, PDGFRA was altered in both treated cells (PC1 and PC2).

Concerning toceranib phosphate targets, we found that *PDGFR-A*, *PDGFR-β*, and *PDGF-D* genes were upregulated in treated PC1 cells and that *PDGFR-A* was downregulated in treated PC2 cells. The list of all upregulated and downregulated genes in treated PC1 cells (supplementary table 1and 2, respectively) and all upregulated and downregulated in treated PC2 cells (supplementary table 3 and 4, respectively).

3.5 Gene Ontology (GO)

We analyzed GO data associated with DEGs in untreated and treated PC1 cells, and we observed 181 common, significantly ($p<0.05$) enriched genes that were upregulated and 82 that were downregulated ($p<0.05$). Redundant GO terms and with no statistically significant difference were removed using Revigo. The analysis revealed that among the 167 biological processes (figure 5A) associated with the upregulated genes in the toceranib phosphate-treated cells, regulation of the PDGFR and PDGFR-β signaling pathway, regulation of the endothelial cell apoptotic process, and negative regulation of vasculature development and morphogenesis of the epithelium were included. A total of 117

biological processes (figure 5C) were associated with the downregulated genes, including regulation of the cell cycle, negative regulation of cell death, sprouting angiogenesis and DNA synthesis involved in DNA repair. These data suggest an important role for toceranib phosphate in the PDGF pathway in PC1 cells and mechanisms related to angiogenesis and cell growth. Thirty-one molecular functions were associated with upregulated genes (figure 5B), and 21 were associated with downregulated genes (figure 5D).

Additionally, when comparing untreated and treated PC2 cells, we observed 18 enriched genes upregulated in treated PC2 cells, from which we found 139 biological processes (figure 6A) and 19 molecular functions (figure 6B), including phosphatidylinositol 3-kinase (PI3-K) signaling, protein serine/threonine kinase activity, mitotic cell cycle regulation and cell migration. In the downregulated genes, 110 biological processes (figure 6C) and 19 molecular functions (figure 6D) were summarized and related to PDGF/PDGFR binding and signaling and regulation of cell motility and proliferation.

3.6 Protein-protein interactions (PPI) network

After removing disconnected nodes in the network, PPI analysis was constructed using upregulated and downregulated genes for each cell line (PC1 and PC2). The analysis comprises a highly interactive PPI network of 170 nodes and 57 interactions in upregulated genes of PC1 cells (figure 7A), 77 nodes and 51 interactions in downregulated genes of PC1 cells (figure 7B), 18 nodes and 3 interactions in upregulated of PC2 cells (figure 7C) and 20 nodes and 1 interaction in downregulated of PC2 cells (figure 7D).

4. Discussion

Despite therapeutic advances, canine prostatic carcinoma is associated with unfavorable prognosis, mainly in metastatic cases, and there is a limited number of studies reporting the efficacy of treatment in canine PC [1,4,7]. Toceranib phosphate has shown efficacy in mast cell tumors and has been reported in different types of canine cancers; however, in the small number of clinical canine PC cases, no molecular mechanism of action has been studied [15,18,24–26].

In the present study, we observed VEGFR2 and PDGFR-β protein expression in two primary canine PC cell lines; these receptors could represent suitable targets for therapy with RTK drugs, including toceranib phosphate. Deregulation of tyrosine kinase receptors is frequently associated with different cancers and metastasis [27–29]. Previously, our research group investigated the KIT expression in canine PC tissue samples and in both cell cultures (PC1 and PC2)[30]. In this study, we identified a heterogenous KIT gene and protein expression among PC tissue samples and no KIT expression was observed in metastatic samples. Besides that, we assessed KIT protein expression by western blot, and we did not find KIT expression in both PC1 and PC2 cells, confirming our immunofluorescence results.

Regarding the PDGFR-β and VEGFR2 expression in canine PC, we previously observed increased VEGFR2 protein expression in formalin-fixed paraffin-embedded canine PCs compared to normal prostate cells, revealing an interesting target for toceranib phosphate (data not published). In humans, VEGFR2 inhibition is associated with reduced osteolysis and growth of prostate carcinoma bone metastasis [31]. To the best of our knowledge, no previous study investigated PDGFR- β in canine PC.

Although we demonstrated the absence of c-KIT protein expression in both canine PC cell lines (PC1 and PC2). We investigated KIT gene expression after TP treatment and no significant difference was observed between the *KIT* transcript levels in treated and untreated (PC1 and PC2) cells. Although c-KIT protein overexpression may be correlated with more aggressive tumors, higher invasion capacity and tumor recurrence, heterogeneity and/or absence of c-KIT protein expression has been demonstrated in the epithelial cells of human prostate cancer [30,32–34]. Moreover, the stromal microenvironment has an important role, and the increased c-KIT expression in stromal cells may affect PC development [35]. In 2D cell culture, we could not verify the role of toceranib phosphate in the stroma. However, in a previous study from our group, using formalin-fixed paraffin-embedded canine PC samples, we observed c-KIT-positive stromal cells [36].

Although both PC1 and PC2 were positive for PDGFR and VEGFR receptors, the toceranib phosphate antitumoral effect was different. After treatment with toceranib phosphate, PC1 cell viability was reduced significantly

($p<0.05$) in a dose-dependent manner, showing drug sensitivity. In contrast, cell viability was not significantly altered in PC2 cells subjected to the same toceranib phosphate concentrations. These data reinforce the intertumor heterogeneity and the importance of prostate cancer treatment planning [37]. Even at higher toceranib phosphate concentrations (data not shown), the PC2 cell line did not show significant cell viability reduction. In addition, if effective, the use of higher concentrations would be out of the maximum dosage for *in vivo* use [38]. Thus, we considered the PC2 cell line resistant to toceranib phosphate treatment.

The global gene expression profile identified differentially expressed genes that can help to explain the resistance mechanism. We found only 17 genes in common between PC1 and PC2 cells comparing untreated and treated, indicating a difference between them after treatment with toceranib phosphate. Considering the differences in global gene analyses between toceranib phosphate-treated and untreated PC1 and PC2 cell lines, we found that *PDGF-D*, *PDGFR-α* and *PDGFR-β* genes, which are involved in the PDGFR pathway, were upregulated in PC1. We confirmed the upregulated expression of *PDGFR-β* in PC1 after treatment by qPCR. Interestingly, both cell lines presented *PDGFR-β* deregulation after treatment; however, PC2 cell line presented *PDGFR-β* downregulation. The increase in RTK-ligand from autocrine tumor cell production and RTK amplification are potential mechanisms of acquired resistance to tyrosine kinase inhibitor [39–41]. Thus, the increased *PDGFR-β* expression in PC1 cells can be related to a direct blockage of its receptor as an attempt to activate *PDGFR-β* pathway even with the use of an inhibitor [42]. On the other hand, PC2 cells showed a *PDGFR- β* downregulation, indicating a direct blockage of this pathway. However, associating this molecular feature with the fact that this tumor cell was resistant to toceranib phosphate, this cell line probably did not present PDGFR- *β* as a driver pathway to tumor proliferation.

PC1 and PC2 cell cultures were previously stablished and characterized by our research group, and both cells are positive for pan-cytokeratin, prostatic specific antigen and negative for androgen receptor (Costa et al. 2019). However, PC1 cell became from a nonmetastatic tumor and PC2 were stablished from a metastatic tumor [21]. Since PC2 cell was originated from a

metastatic tumor, it is expected a higher number of gene alterations in this cell line, that can lead with tumor resistance.

Besides that, we observed increase in VEGFR2 in PC2 after toceranib phosphate treatment by qPCR, however no difference was observed in microarray analysis, but qPCR can detect small signal differences undetectable by microarrays due to its flexible number of cycles [43]. In addition, *PDGF-D* seems to activate *PDGFR-β* without the involvement of its classical ligand, *PGDF-B*. Additionally, increased transcription of these genes has been related to tumor aggressiveness and epithelial-mesenchymal transition, including in prostate cancer [44–46].

Differentially expressed genes involved in negative regulation of vasculature development and blood vessel endothelial cell proliferation, which are related to a decrease in sprouting angiogenesis, were identified in treated PC1 cells. Moreover, toceranib phosphate significantly affected genes involved in cell growth and activation and DNA repair by different mechanisms, for example, by decreasing the mitotic cell cycle and cellular response to DNA damage stimuli. As would be expected of an inhibitor of VEGFR, PDGFR and c-KIT kinase activity, toceranib phosphate can modify the expression of genes related to antiangiogenic activity and tumor growth inhibition via direct and indirect mechanisms [11,14].

Another explanation for PC2 resistance to toceranib phosphate could be based on the upregulation of genes from the PI3K pathway, which positively regulates the mitotic cell cycle, protein serine/threonine kinase activity and cell migration. Therefore, treated PC2 cells may become resistant by activating parallel signaling pathways and mechanisms.

We found overexpression of *CEMIP* (cell migration inducing protein), described as an activator of PI3K signaling [47]. Dysregulated and/or elevated activation of the PI3K signaling network is one of the most common events in the oncogenesis of different cancers, including prostate cancer [48–50]. In human prostate cancer, inhibition of PI3K signaling has been demonstrated to suppress invasion and induce apoptosis [51]. Additionally, *CEMIP* plays a critical role in cancer cell migration and invasion and is negatively correlated with patient survival [52,53].

Consistent with the main mechanism of action of toceranib phosphate, our results showed downregulation of genes involved in transmembrane receptor protein tyrosine kinase activity and PDGFR binding. The role of negative feedback in growth factor signaling pathways is to generate stability, limiting the duration and extent of signaling and preventing potentially toxic overactivation of signaling [54]. However, we found overexpression of endothelin-1 (*ET-1*), which can be a compensatory cellular mechanism that acts alone or in cooperation with other tyrosine kinase growth factors, such as PDGFR and VEGFR, to activate TK receptors leading to cell proliferation and angiogenesis [55–58]. Signaling pathways require feedback loops that are positive and negative to adjust their cellular response through different genes, mechanisms and stimuli [59]. Therefore, loss of homeostasis of this negative feedback can lead to oncogene activation and uncontrolled growth that can lead to cancer [60].

Migration and invasion are fundamental characteristics of cancer progression and metastasis that consequently reduce therapy efficacy and prognosis. This study identified the overexpression of genes involved in these mechanisms, such as *RND1* (Rho family GTPase 1) and *SEMA3D* (Semaphorin 3D). RND1 protein is concentrated at adherens junctions in both confluent fibroblasts and epithelial cells, and overexpression causes loss of cell-matrix adhesion, leading to cell rounding which facilitates invasion [61]. SEMA3D, a membrane bound protein, is involved in cell-cell communication and plays an important role in many pathophysiological processes, such as cancers, and its overexpression has been related to increased cell invasiveness [62,63].

On the other hand, other genes involved in metastatic processes, such as *MMP1* and *MMP3,* were downregulated. Matrix metalloproteinases (MMPs) are capable of cleaving extracellular matrix protein substrates and have been identified as key factors involved in carcinogenesis and metastasis [64,65]. Among the members of the MMP family, MMP1 degrades fibrillary collagen and has been associated with invasion and poor prognosis [66]. The *MMP3* gene is also implicated as a contributor to cancer progression, responsible for inducing epithelial-mesenchymal transition and increasing cell spreading [67,68]. Our PPI network indicated an interaction between MMP1 and MMP3, which suggests a relationship between both genes and an important role in canine PC.

Interestingly, we found downregulation of *EFEMP1* (epidermal growth factor-containing fibulin-like extracellular matrix protein 1), which encodes a member of the fibulin family of secreted glycoproteins, and its role in carcinogenesis is controversial due to both suppressive and oncogenic activities [69]. In the serum and urine of prostate cancer patients, decreased expression of EFEMP1 was reported, suggesting that this protein participates in the carcinogenesis of human PC [70].

In summary, we demonstrated that two different primary canine prostate cancer cell lines have similar patterns of TK receptor protein expression but have different responses to TK inhibition treatment with toceranib phosphate. Based on global gene comparative analysis, this study revealed that toceranib phosphate could affect different genes involved in the progression of canine PC. These DEGs are important for investigating other mechanisms involved in RTK therapy and are useful targets for treating canine PC. Moreover, DEGs associated with mechanisms of cancer drug resistance were observed, raising concern that drug resistance may develop. These findings support the biological response in some cases of canine PC and the need for more personalized cancer treatments.

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Author contributions

P.E.K. executed some of the experiments, analyzed the data, prepared the figures and wrote the manuscript. P.F.L., A.F.L.F. and M.C. performed the experiments and collected data. F.K.D. and R.F.C. analyzed and interpretation of data. C.E.F.A. conceived the project, helped with data collection, interpretation of data. R.L.A. conceived the project, participated in its design and its coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

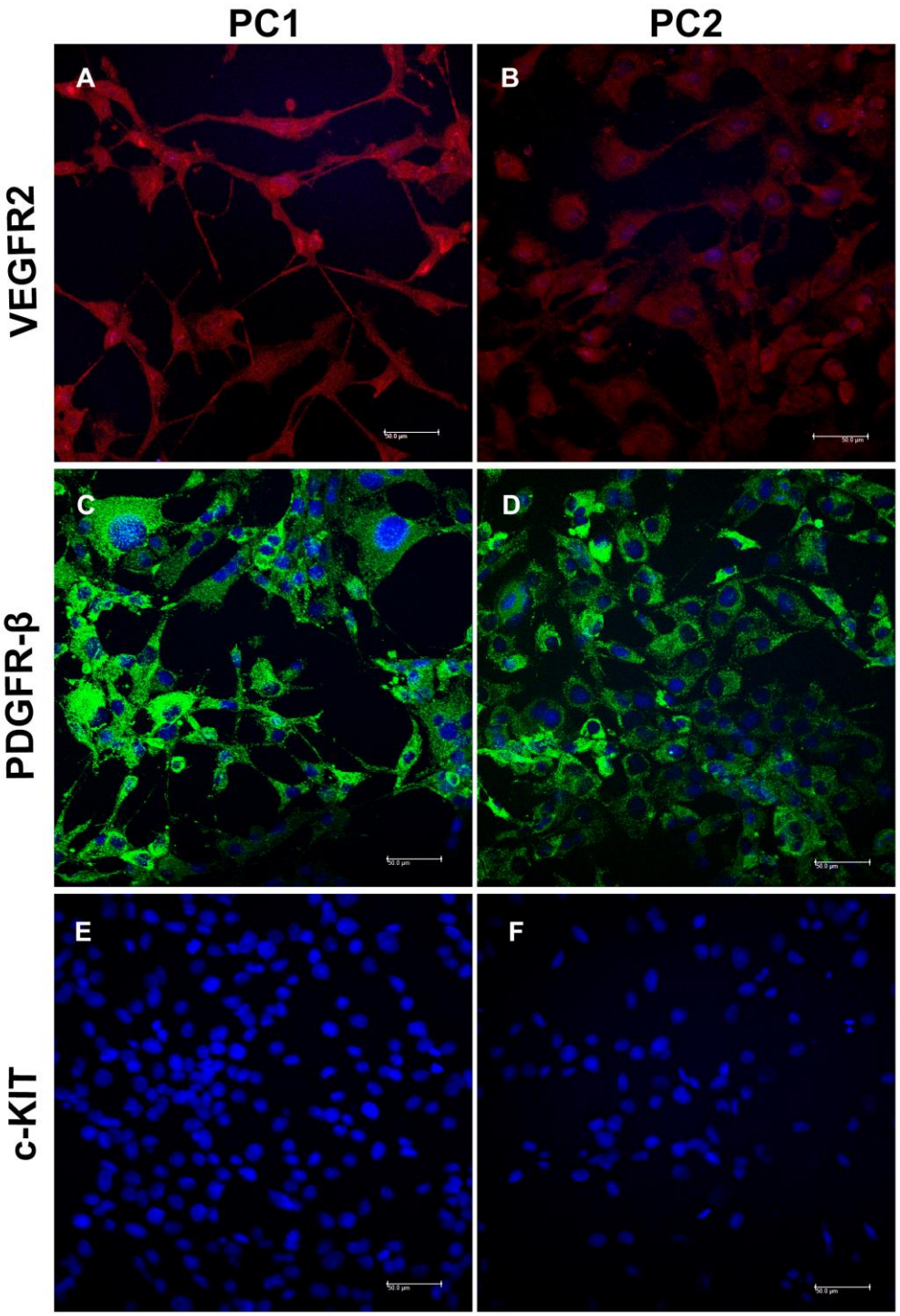

Figure 1: Detection of VEGFR2, PDGFR-β and c-KIT protein expression by immunofluorescence. Cytoplasmic expression of VEGFR2 in red (A and B) and PDGFR-β in green (C and D) in PC1 and PC2 samples, respectively. No c-KIT protein expression (E and F) was observed in both cells. The nuclei were counterstained with DAPI (blue).

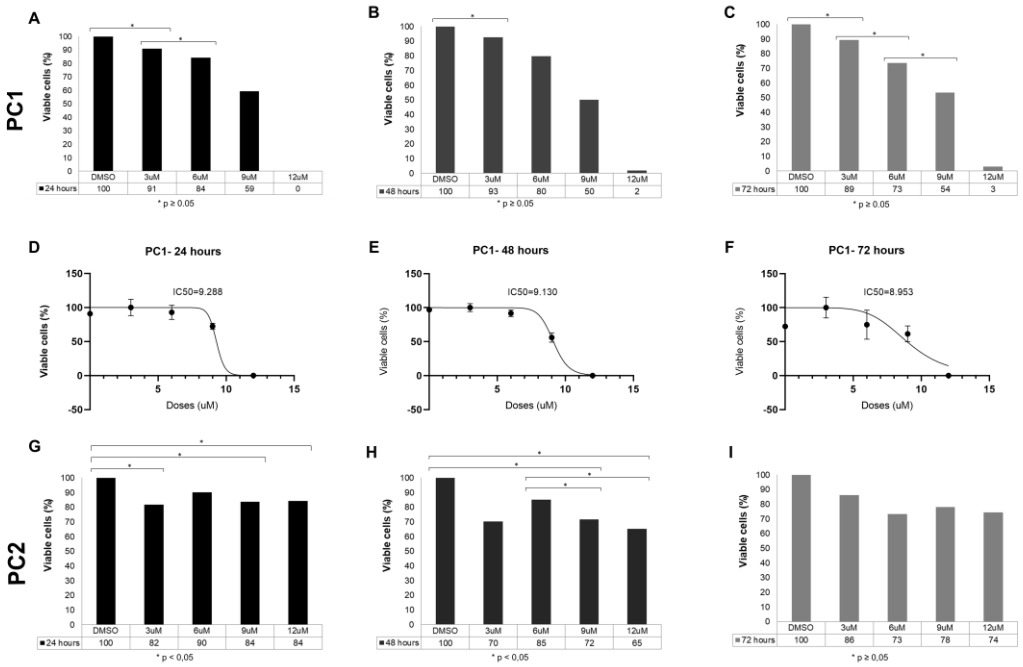

Figure 2: PC1 and PC2 cells were cultured with various concentrations of toceranib phosphate (3 µM, 6 µM, 9 µM and 12 µM) and the degree of cell viability was assessed using an MTT assay. PC1 cells were treated with toceranib phosphate for (A) 24, (B) 48 and (C) 72 hours. IC_{50} values of toceranib phosphate after (D) 24, (E) 48 and (F) 72 hours incubation in PC1 cells. PC2 cells were treated with toceranib phosphate for (G) 24, (H) 48 and (I) 72 hours. A value of $p<0.05$ was considered statistically significant.

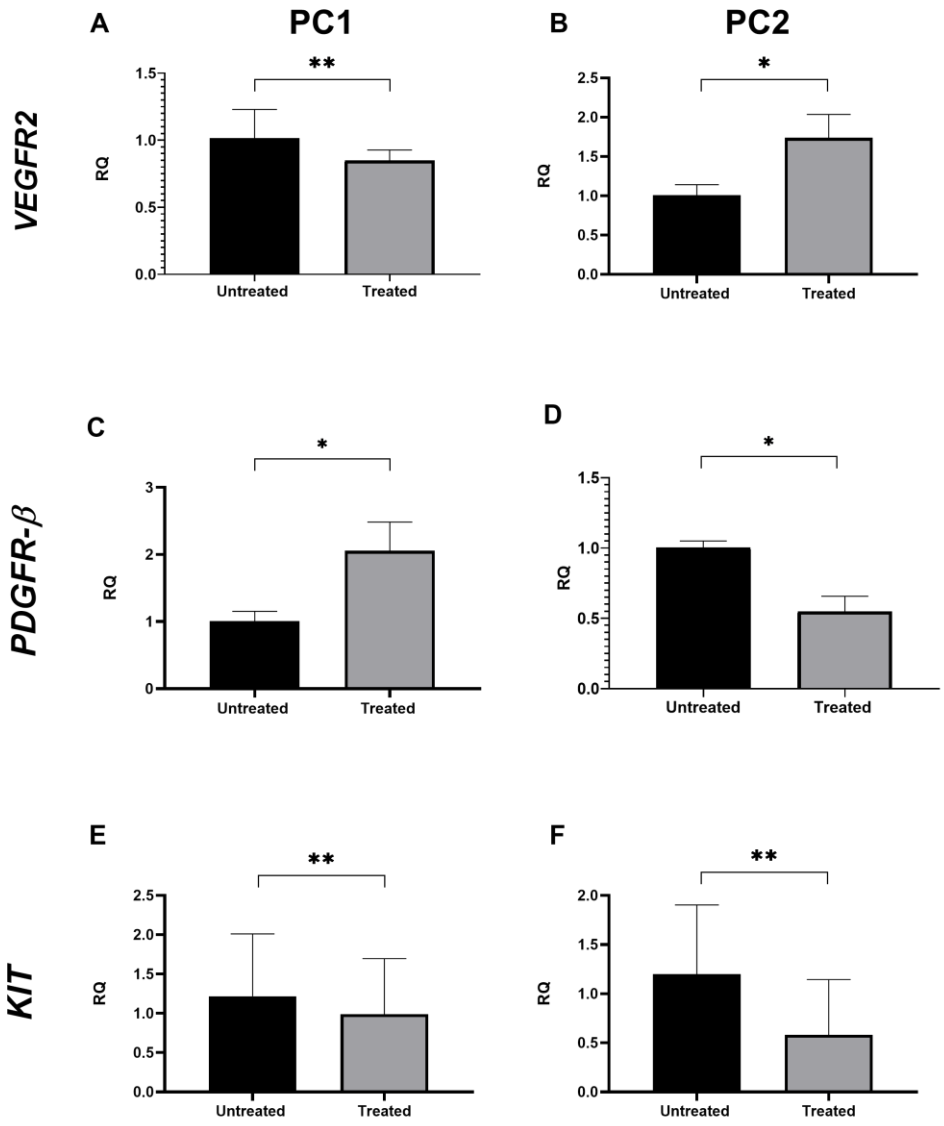

Figure 3: The effect of toceranib phosphate on *VEGFR2*, *PDGFR-β* and *KIT* transcript levels in PC1 and PC2 cells. Relative quantification levels of *VEGFR2* (A and B), *PDGFR- β* (C and D) and *KIT* (E and F) were measured by RT-qPCR in PC1 and PC2 cells treated and untreated (DMSO) with toceranib phosphate (IC50 value). *= $p < 0.05$, ** = $p \geq 0.05$.

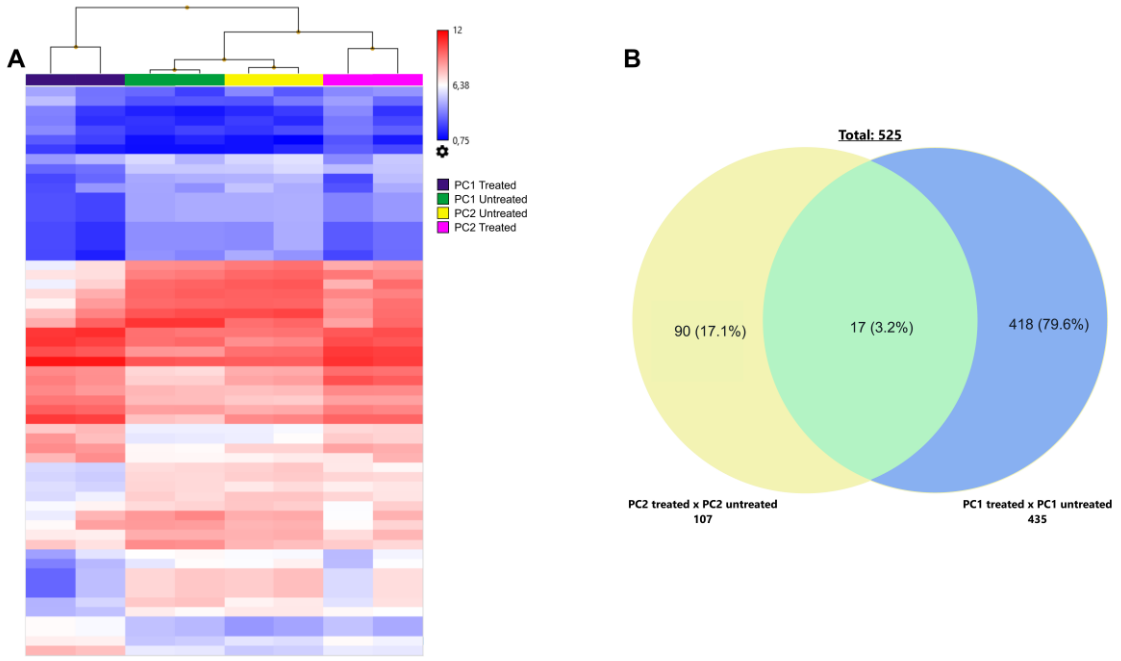

Figure 4: Differentially expressed genes in untreated and treated canine PC1 and PC2 cell lines with toceranib phosphate based on microarray analysis. (A) Cluster heatmap: red represents upregulation while green indicates downregulation of gene expression relative to untreated cells. Each row represented a gene and each column represented a sample. Each sample was analyzed in duplicate. (B) Venn diagram: overlapping section show common genes deregulated by toceranib phosphate in PC1 (yellow) and PC2 (blue) cells treated compared to untreated.

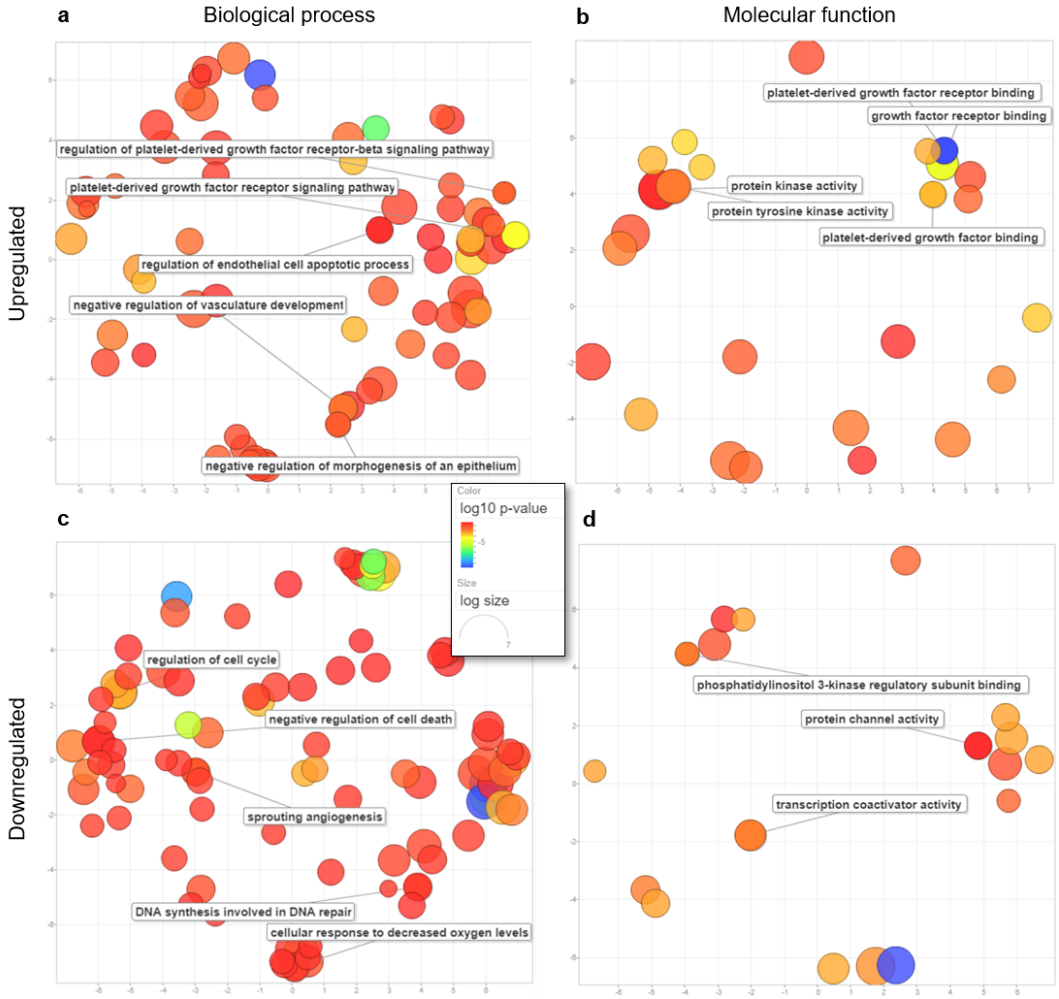

Figure 5: Gene ontology processes: common biological processes and molecular functions in both upregulated and downregulated DEGs in treated PC1 cells. (a) Biological processes in upregulated DEGs, (b) molecular functions in upregulated DEGs, (c) biological processes in downregulated DEGs, (d) molecular functions in downregulated DEGs. Scatterplot obtained by REVIGO software.

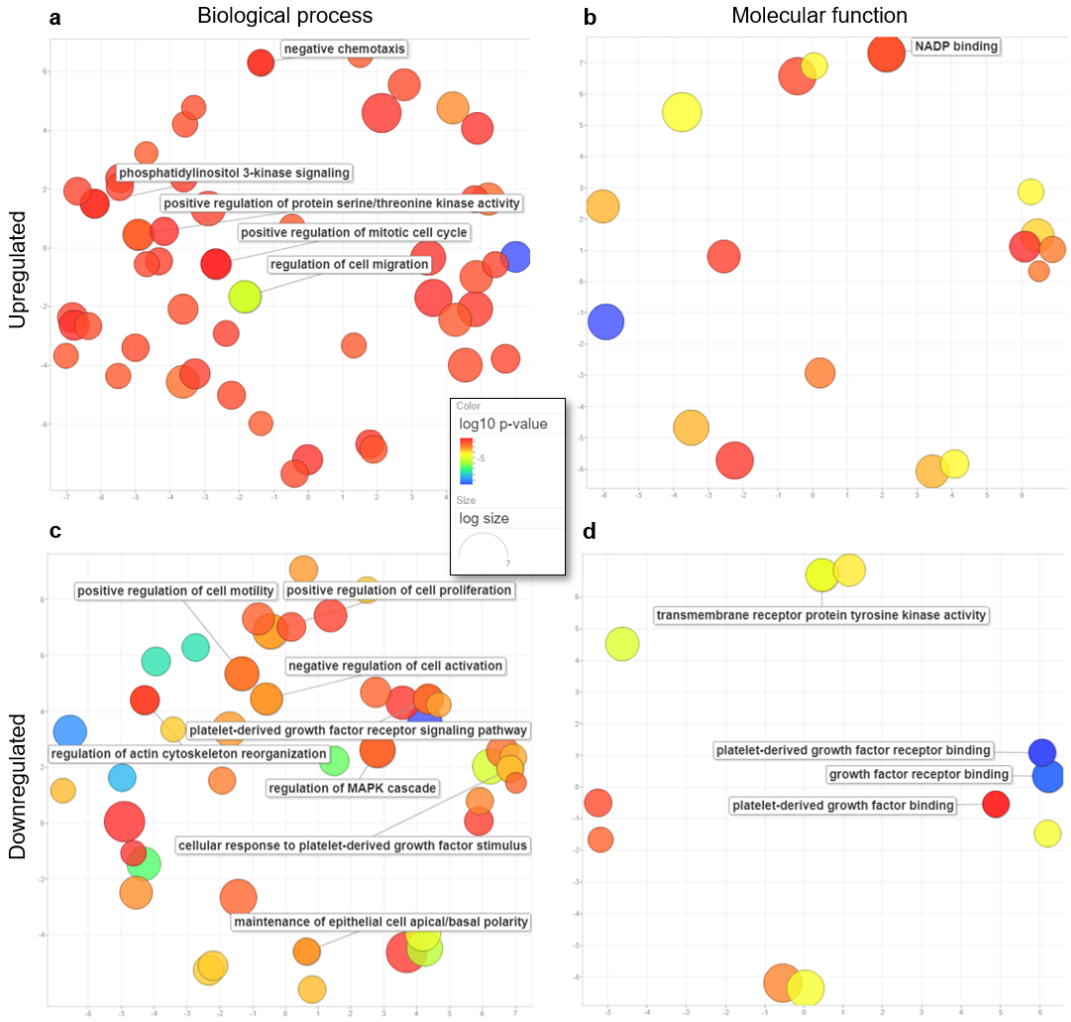

Figure 6: Gene ontology processes: common biological processes and molecular functions in both upregulated and downregulated DEGs in treated PC2 cells. (a) Biological processes in upregulated DEGs, (b) molecular functions in upregulated DEGs, (c) biological processes in downregulated DEGs, (d) molecular functions in downregulated DEGs. Scatterplot obtained by REVIGO software.

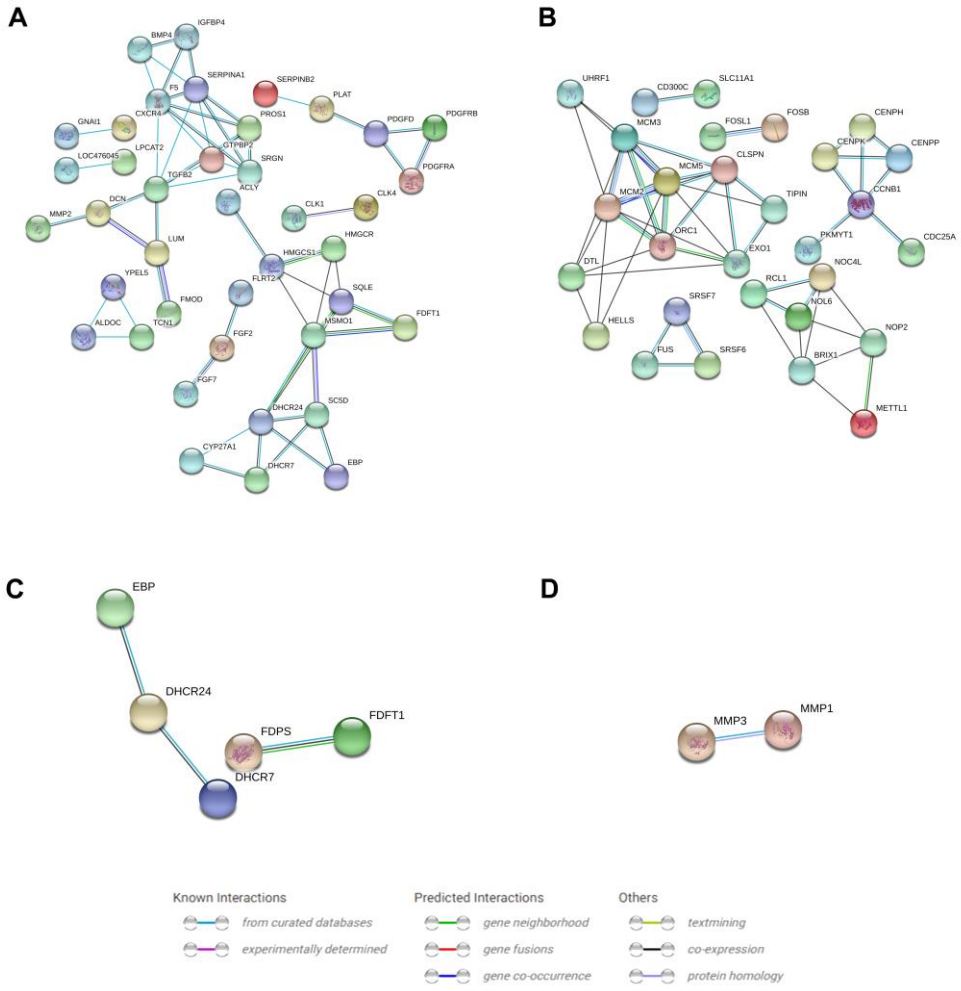

Figure 7: Gene interaction network of upregulated differentially expressed genes in treated PC1 (A) and PC2 (C) cells and downregulated DEGs in treated PC1 (B) and PC2 cells (D) identified by STRING software using a high confidence interaction score (0.700). Circles represent genes, lines represent protein-protein associations.

Supplementary informations

Supplementary table S1: Upregulated genes in treated PC1 cells

Gene Symbol	Gene Name	Entrez ID	FC	p value
INSIG1	insulin induced gene 1	14300195	7,59	0,00000576
NRXN1	neurexin 1	14272951	7,69	0,0000284
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	14404882	4,56	0,0000306
PLA2G16	phospholipase A2, group XVI	14312826	3,57	0,0000466
TFPI2	tissue factor pathway inhibitor 2	14292597	3,69	0,0000587
DCN	decorin	14298259	3,67	0,0000658
SLCO5A1	solute carrier organic anion transporter family, member 5A1	14373210	3,02	0,000068
SLC24A2	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	14277552	3,06	0,0000708
ZNF608	zinc finger protein 608	14276650	3,04	0,0000738

RASL11A	RAS-like, family 11, member A	14355340	3,44	0,0000746
DOPEY2	dopey family member 2	14384800	3,18	0,000076
LUM	lumican	14298255	8,07	0,0000867
FDFT1	farnesyl-diphosphate farnesyltransferase 1	14355799	3,02	0,000098
LDLR	low density lipoprotein receptor	14335961	2,64	0,0001
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	14379074	2,67	0,0001
EBP	emopamil binding protein (sterol isomerase)	14458384	2,68	0,0001
BMP4	bone morphogenetic protein 4	14441454	2,7	0,0001
HBP1	HMG-box transcription factor 1	14313517	2,77	0,0001
LOC490172	interferon-induced guanylate-binding protein 1	14423593	2,77	0,0001
KLHL24	kelch-like family member 24	14391611	2,84	0,0001
BMF	Bcl2 modifying factor	14381834	2,88	0,0001
TCP11L2	t-complex 11, testis-specific-like 2	14272077	3,04	0,0001
AEBP1	AE binding protein 1	14456550	3,05	0,0001
MOXD1	monooxygenase, DBH-like 1	14261282	3,44	0,0001
NCALD	neurocalcin delta	14287630	3,7	0,0001
SULF1	sulfatase 1	14372174	2,31	0,0002
TP53INP1	tumor protein p53 inducible nuclear protein 1	14373699	2,33	0,0002
FLRT2	fibronectin leucine rich transmembrane protein 2	14438980	2,36	0,0002
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	14442716	2,36	0,0002
MMP2	matrix metallopeptidase 2	14325400	2,49	0,0002
CCNG2	cyclin G2	14386540	2,52	0,0002
ZC3H6	zinc finger CCCH-type containing 6	14304983	2,53	0,0002
ENPP5	ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative)	14283727	2,54	0,0002
LOC479476	arachidonate 12-lipoxygenase, 12S-type	14410212	2,54	0,0002
ITGA10	integrin, alpha 10	14309269	2,56	0,0002
ARRDC3	arrestin domain containing 3	14374105	2,56	0,0002
TGM2	transglutaminase 2	14351635	2,57	0,0002
GTPBP2	GTP binding protein 2	14283638	2,66	0,0002
NFIL3	nuclear factor, interleukin 3 regulated	14257734	2,76	0,0002
LOC491477	carcinoembryonic antigen-related cell adhesion molecule 21	14259697	3,2	0,0002
LIPG	lipase, endothelial	14436583	3,74	0,0002
LPIN1	lipin 1	14303706	2,18	0,0003
IGFBP4	insulin-like growth factor binding protein 4	14451427	2,24	0,0003
PDGFR-A	platelet-derived growth factor receptor, alpha polypeptide	14286984	2,25	0,0003
ACSL4	acyl-CoA synthetase long-chain family member 4	14463411	2,25	0,0003
LPCAT2	lysophosphatidylcholine acyltransferase 2	14325383	2,25	0,0003
YPEL4	yippee-like 4	14311136	2,26	0,0003
GFRA1	GDNF family receptor alpha 1	14371215	2,3	0,0003
RGS2	regulator of G-protein signaling 2	14401712	2,34	0,0003
TXNIP	thioredoxin interacting protein	14309321	2,38	0,0003
GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	14310560	2,38	0,0003
CLK1	CDC-like kinase 1	14399459	2,44	0,0003
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	14377077	2,48	0,0003

NIPAL1	NIPA-like domain containing 1	14286865	2,58	0,0003
SQLE	squalene epoxidase	14286196	2,63	0,0003
JCHAIN	joining chain of multimeric IgA and IgM	14289740	2,65	0,0003
DHCR7	7-dehydrocholesterol reductase	14311987	2,66	0,0003
IL1R1	interleukin 1 receptor, type I	14272400	2,67	0,0003
SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	14294876	2,74	0,0003
SLC24A2	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2	14277542	3,24	0,0003
SVIL	supervillin	14323596	2,14	0,0004
ACLY	ATP citrate lyase	14445439	2,15	0,0004
PDGFR-B	platelet-derived growth factor receptor, beta polypeptide	14404677	2,2	0,0004
CERCAM	cerebral endothelial cell adhesion molecule	14454960	2,21	0,0004
ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	14392983	2,23	0,0004
PLAT	plasminogen activator, tissue	14300372	2,25	0,0004
PLAG1	pleiomorphic adenoma gene 1	14372951	2,3	0,0004
DDIT4	DNA-damage-inducible transcript 4	14403416	2,36	0,0004
TLR1	toll-like receptor 1	14375983	2,37	0,0004
SUGCT	succinyl-CoA:glutarate-CoA transferase	14313399	2,41	0,0004
EXD1	exonuclease 3-5 domain containing 1	14381939	2,41	0,0004
ARHGAP20	Rho GTPase activating protein 20	14409613	2,69	0,0004
DHCR24	24-dehydrocholesterol reductase	14411672	2,76	0,0004
NEIL2	nei-like DNA glycosylase 2	14355811	2,78	0,0004
NR4A2	nuclear receptor subfamily 4, group A, member 2	14396194	2,82	0,0004
ALDOC	aldolase C, fructose-bisphosphate	14453038	2,02	0,0005
NDRG2	NDRG family member 2	14297825	2,02	0,0005
FGF2	fibroblast growth factor 2 (basic)	14318122	2,05	0,0005
SORBS1	sorbin and SH3 domain containing 1	14370119	2,06	0,0005
KDM7A	lysine (K)-specific demethylase 7A	14299497	2,1	0,0005
C5H11orf70	chromosome 5 open reading frame, human C11orf70	14415409	2,11	0,0005
CRLF1	cytokine receptor-like factor 1	14329880	2,15	0,0005
CALCOCO1	calcium binding and coiled-coil domain 1	14362391	2,31	0,0005
CREBRF	CREB3 regulatory factor	14407191	2,57	0,0005
DCLK1	doublecortin-like kinase 1	14352910	2,58	0,0005
CXCR4	chemokine (C-X-C motif) receptor 4	14318722	2,79	0,0005
GFRA1	GDNF family receptor alpha 1	14371208	2,82	0,0005
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	14384142	3,45	0,0005
CCPG1	cell cycle progression 1	14383106	2,01	0,0006
ADGRD1	adhesion G protein-coupled receptor D1	14359850	2,02	0,0006
SREBF2	sterol regulatory element binding transcription factor 2	14271290	2,02	0,0006
YPEL5	yippee-like 5	14304384	2,02	0,0006
SLC6A13	solute carrier family 6 (neurotransmitter transporter), member 13	14367286	2,05	0,0006
OPTN	optineurin	14323843	2,14	0,0006
KLHDC1	kelch domain containing 1	14437504	2,17	0,0006
DKK3	dickkopf WNT signaling pathway inhibitor 3	14341158	2,18	0,0006
LAMC2	laminin, gamma 2	14430058	2,24	0,0006

RBL2	retinoblastoma-like 2	14325432	2,25	0,0006
PRSS50	protease, serine, 50	14329557	2,31	0,0006
FBXO32	F-box protein 32	14288111	2,44	0,0006
PID1	phosphotyrosine interaction domain containing 1	14356604	2,01	0,0007
PSD3	pleckstrin and Sec7 domain containing 3	14302256	2,04	0,0007
ACSS3	acyl-CoA synthetase short-chain family member 3	14295292	2,37	0,0007
FAM78B	family with sequence similarity 78, member B	14400913	2,46	0,0007
KLF9	Kruppel-like factor 9	14257441	2,64	0,0007
SRGN	serglycin	14403177	2,65	0,0007
LOC479922; LOC607314	pancreatic alpha-amylase; pancreatic alpha-amylase-like	14456388	2,68	0,0007
KANSL1L	KAT8 regulatory NSL complex subunit 1-like	14399811	2,03	0,0008
FBXL2	F-box and leucine-rich repeat protein 2	14346079	2,04	0,0008
NFE2L1	nuclear factor, erythroid 2-like 1	14446155	2,05	0,0008
GSAP	gamma-secretase activating protein	14313677	2,08	0,0008
CHAC1	ChaC glutathione-specific gamma-glutamylcyclotransferase 1	14379835	2,12	0,0008
FAXDC2	fatty acid hydroxylase domain containing 2	14404448	2,39	0,0008
TGFB2	transforming growth factor, beta 2	14400771	2,52	0,0008
SC5D	sterol-C5-desaturase	14414360	2,54	0,0008
ACP5	acid phosphatase 5, tartrate resistant	14330808	2,54	0,0008
MIR29C-1; MIR29C-2	microRNA mir-29c-1; microRNA mir-29c-2	14433548	2,55	0,0008
MIR29C-1; MIR29C-2	microRNA mir-29c-1; microRNA mir-29c-2	14433550	2,55	0,0008
SYTL2	synaptotagmin-like 2	14337697	2,73	0,0008
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	14368790	2,03	0,0009
DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	14428812	2,09	0,0009
MIR8810; AMPD3	microRNA mir-8810; adenosine monophosphate deaminase 3	14338927	2,12	0,0009
BCL6	B-cell CLL/lymphoma 6	14393213	2,18	0,0009
JMY	junction mediating and regulatory protein, p53 cofactor	14376901	2,23	0,0009
STK38L	serine/threonine kinase 38 like	14366257	2,26	0,0009
TCN1	transcobalamin I (vitamin B12 binding protein, R binder family)	14341603	2,29	0,0009
MUC15	mucin 15, cell surface associated	14341505	2,06	0,0011
FUT1	fucosyltransferase 1 (galactoside 2-alpha-L-fucosyltransferase, H blood group)	14259031	2,08	0,0011
SAT1	spermidine/spermine N1-acetyltransferase 1	14457690	2,59	0,0011
EDNRA	endothelin receptor type A	14296014	2,74	0,0011
MAML3	mastermind-like transcriptional coactivator 3	14316870	2,04	0,0012
ZNF395	zinc finger protein 395	14353660	2,11	0,0012
SELL	selectin L	14430659	2,99	0,0012
CLK4	CDC-like kinase 4	14273866	2,24	0,0013
CERS3	ceramide synthase 3	14374679	2,42	0,0014
ERAP2	endoplasmic reticulum aminopeptidase 2	14376482	2,89	0,0014
FABP3	fatty acid binding protein 3, muscle and heart	14321832	2,05	0,0015
SMOC2	SPARC related modular calcium binding 2	14256513	2,06	0,0015
SGK494	uncharacterized serine/threonine-protein kinase SgK494	14453076	2,01	0,0017
SLC16A2	solute carrier family 16, member 2 (thyroid hormone transporter)	14459088	2,21	0,0017
LOC612019;	pancreatic alpha-amylase; pancreatic alpha-amylase-like	14455986	2,35	0,0017

LOC479922; LOC480825; LOC607314				
PDGF-D	platelet derived growth factor D	14409780	2,47	0,0017
DLA-DRA	MHC class II DR alpha chain	14279695	2,93	0,0018
PCDH7	protocadherin 7	14379107	2,09	0,0019
FGF7	fibroblast growth factor 7	14380425	2,68	0,0019
KLHL15	kelch-like family member 15	14461586	2,18	0,002
FMOD	fibromodulin	14401381	2,03	0,0021
FAT4	FAT atypical cadherin 4	14318073	2,04	0,0022
LOC607276; LOC612019	pancreatic alpha-amylase-like; pancreatic alpha-amylase	14455969	2,44	0,0022
DHRS2	dehydrogenase/reductase (SDR family) member 2	14436903	2,07	0,0025
F5	coagulation factor V (proaccelerin, labile factor)	14430684	2,09	0,0025
SUGCT	succinyl-CoA:glutarate-CoA transferase	14313394	2,04	0,0026
SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	14447713	2,71	0,0026
RSRP1	arginine/serine-rich protein 1	14322184	2,14	0,0027
MCOLN3	mucolipin 3	14423752	2,33	0,0027
ELOVL7	ELOVL fatty acid elongase 7	14324780	2,3	0,003
LOC100685565	tetratricopeptide repeat protein 39B-like	14304668	2,35	0,003
LOC607460; LOC612019	pancreatic alpha-amylase	14456239	2,55	0,003
LOC102154725	methylsterol monooxygenase 1 pseudogene	14457299	3,4	0,0031
FAM35A	family with sequence similarity 35, member A	14403840	2,06	0,0032
CHL1	cell adhesion molecule L1-like	14333480	2,49	0,0034
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	14255559	2,03	0,0035
TESK2	testis-specific kinase 2	14294737	2,1	0,0036
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	14260883	2,27	0,0037
PDE3A	phosphodiesterase 3A, cGMP-inhibited	14366416	2,35	0,0041
CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1	14398789	2,35	0,005
CALCRL	calcitonin receptor-like	14397517	2,12	0,0051
LRCH2	leucine-rich repeats and calponin homology (CH) domain containing 2	14463516	2,04	0,0052
VLDLR	very low density lipoprotein receptor	14257570	2,02	0,0055
LOC100855970	protocadherin beta-4	14320227	2,01	0,006
RARRES3	retinoic acid receptor responder (tazarotene induced) 3	14316455	2,28	0,0063
CFAP69	cilia and flagella associated protein 69	14290438	2,03	0,0067
MIR125B-1	microRNA mir-125b-1	14409053	2,48	0,0072
KLHL28	kelch-like family member 28	14441087	2,28	0,0075
MTURN	maturin, neural progenitor differentiation regulator homolog (Xenopus)	14291266	2,12	0,0078
PROS1	protein S (alpha)	14389903	2,31	0,0082
GLRB	glycine receptor, beta	14296277	2,45	0,0084
MPPED2	metallophosphoesterase domain containing 2	14310985	2,02	0,0101
CDS1	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 1	14386750	2,05	0,0152
OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	14364302	2,03	0,0157
MEST	mesoderm specific transcript	14292024	2,36	0,0161
OR10A12	olfactory receptor	14330388	2,13	0,0166
DHRS1	dehydrogenase/reductase (SDR family) member 1	14440617	2,05	0,0169

LOC100682624	olfactory receptor 4C11-like	14456199	2,12	0,042

Supplementary table S2: Downregulated genes in treated PC1 cells

Gene Symbol	Gene Name	Entrez ID	FC	p value
LOC612564	membrane-spanning 4-domains subfamily A member 7	14339519	-3,55	5,05E-05
SRSF6	serine/arginine-rich splicing factor 6	14349213	-3,2	8,88E-05
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	14348293	-2,79	0,0001
CCNB1	cyclin B1	14320839	-2,54	0,0001
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	14278443	-3,31	0,0002
CLDN10	claudin 10	14342285	-2,6	0,0002
SPINK5	serine peptidase inhibitor, Kazal type 5	14320425	-3,17	0,0003
LOC606761	cell division cycle-associated protein 7	14354874	-2,51	0,0003
RANBP1	RAN binding protein 1	14361940	-2,18	0,0003
CDCA7	cell division cycle associated 7	14395825	-3,26	0,0004
LOC485710	uncharacterized LOC485710	14347297	-3,17	0,0004
VGLL2	vestigial-like family member 2	14256601	-2,86	0,0004
SBSPON	somatomedin B and thrombospondin, type 1 domain containing	14373323	-2,68	0,0004
CD3EAP	CD3e molecule, epsilon associated protein	14264838	-2,3	0,0004
FOSL1	FOS-like antigen 1	14312391	-2,29	0,0004
FUS	FUS RNA binding protein	14425550	-2,26	0,0004
METTL1	methyltransferase like 1	14270434	-2,23	0,0004
RNASEH2B	ribonuclease H2, subunit B	14342861	-2,11	0,0004
E2F1	E2F transcription factor 1	14351236	-2,07	0,0004
GINS3	GINS complex subunit 3 (Psf3 homolog)	14325046	-2,48	0,0005
LOC100688091	uncharacterized LOC100688091	14312880	-2,19	0,0005
MRTO4	MRT4 homolog, ribosome maturation factor	14326635	-2,1	0,0005
SASS6	SAS-6 centriolar assembly protein	14423255	-2,06	0,0005
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	14369962	-4,16	0,0006
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	14341081	-2,21	0,0006
DTD2	D-tyrosyl-tRNA deacylase 2 (putative)	14440870	-2,15	0,0007
TOMM40	translocase of outer mitochondrial membrane 40 homolog (yeast)	14264954	-2,1	0,0007
NAF1	nuclear assembly factor 1 ribonucleoprotein	14299058	-2,02	0,0007
TIPIN	TIMELESS interacting protein	14383783	-2,02	0,0007
TIGAR	TP53 induced glycolysis regulatory phosphatase	14367167	-2,23	0,0008
E2F8	E2F transcription factor 8	14341467	-2,4	0,0009
LOC609800	CMRF35-like molecule 6	14444226	-2,01	0,0009
CENPP	centromere protein P	14263476	-2,2	0,001
UNG	uracil DNA glycosylase	14361237	-3,12	0,0011
FAM83A	family with sequence similarity 83, member A	14286103	-2,02	0,0011
QTRTD1	queuine tRNA-ribosyltransferase domain containing 1	14389366	-2,2	0,0013
SRSF7	serine/arginine-rich splicing factor 7	14307855	-2,13	0,0013
POLR3F	polymerase (RNA) III (DNA directed) polypeptide F, 39 kDa	14350460	-2,03	0,0014

NPY	neuropeptide Y	14291140	-2,13	0,0015
TMEM201	transmembrane protein 201	14412387	-2,23	0,0016
CABYR	calcium binding tyrosine-(Y)-phosphorylation regulated	14435923	-2,16	0,0016
CDC25A	cell division cycle 25A	14329359	-2,38	0,0017
ESM1	endothelial cell-specific molecule 1	14404773	-2,78	0,0019
SLC11A1	solute carrier family 11 (proton-coupled divalent metal ion transporter), member 1	14398682	-2,71	0,0019
LOC484356	uncharacterized LOC484356	14258668	-2,22	0,0021
CENPK	centromere protein K	14324857	-2,4	0,0022
GEM	GTP binding protein overexpressed in skeletal muscle	14373638	-2,13	0,0022
HMOX1	heme oxygenase 1	14271904	-2,62	0,0023
AMD1	adenosylmethionine decarboxylase 1	14282088	-2,03	0,0023
PKMYT1	protein kinase, membrane associated tyrosine/threonine 1	14422314	-2,18	0,0025
DTL	denticleless E3 ubiquitin protein ligase homolog (Drosophila)	14429668	-2,01	0,0026
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	14264833	-2,47	0,0027
WNT2B	wingless-type MMTV integration site family, member 2B	14309822	-2,24	0,0029
CEP55	centrosomal protein 55kDa	14367893	-2,17	0,0033
CENPH	centromere protein H	14320843	-2,36	0,004
SPP1	secreted phosphoprotein 1	14386892	-2,24	0,004
OGN	osteoglycin	14257864	-2,09	0,004
NOC4L	nucleolar complex associated 4 homolog	14359746	-2,7	0,0043
EXO1	exonuclease 1	14430897	-2,33	0,0043
NOP2	NOP2 nucleolar protein	14364687	-2,23	0,0044
UHRF1	ubiquitin-like with PHD and ring finger domains 1	14336719	-2,55	0,0045
NOL6	nucleolar protein 6 (RNA-associated)	14277793	-2,12	0,0052
ORC1	origin recognition complex, subunit 1	14294361	-2,48	0,006
HNRNPDL	heterogeneous nuclear ribonucleoprotein D-like	14387831	-2,41	0,0064
RCL1	RNA terminal phosphate cyclase-like 1	14257624	-2,24	0,0065
PSMC3IP; FAM134C	PSMC3 interacting protein; family with sequence similarity 134, member C	14445295	-2,09	0,0065
SKA3	spindle and kinetochore associated complex subunit 3	14353285	-2,05	0,0068
WFDC5	WAP four-disulfide core domain 5	14351820	-2,02	0,0068
BRIX1	BRX1, biogenesis of ribosomes	14408426	-2,19	0,0072
MCM2	minichromosome maintenance complex component 2	14327242	-2,6	0,0073
MIR98	microRNA mir-98	14462430	-2,44	0,0073
LOC486670	histone H4	14364118	-2,12	0,0079
MCM5	minichromosome maintenance complex component 5	14271891	-2,87	0,0082
LOC486484	olfactory receptor 6C75-like	14365165	-2,02	0,0084
CLSPN	claspin	14294119	-2,19	0,0102
SLC25A19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	14444093	-2,09	0,0102
PIGW	phosphatidylinositol glycan anchor biosynthesis, class W	14446944	-2,02	0,0134
HELLS	helicase, lymphoid-specific	14367993	-2,17	0,0147
TRIP13	thyroid hormone receptor interactor 13	14392722	-2,08	0,0166
MCM3	minichromosome maintenance complex component 3	14283972	-2,09	0,0184
HSPE1	heat shock 10kDa protein 1 (chaperonin 10)	14397790	-2,02	0,0205

AIMP1	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1	14387277	-2,08	0,0279

Supplementary table S3: Upregulated genes in treated PC2 cells

Gene Symbol	Gene Name	Entrez ID	FC	p value
DHCR24	24-dehydrocholesterol reductase	14411672	3,2	8,45E-05
NEXN	nexilin (F actin binding protein)	14428818	2,84	0,0002
FDFT1	farnesyl-diphosphate farnesyltransferase 1	14355799	2,58	0,0002
EDN1	endothelin 1	14393998	2,54	0,0002
DHCR7	7-dehydrocholesterol reductase	14311987	2,19	0,0002
FABP3	fatty acid binding protein 3, muscle and heart	14321832	2,41	0,0003
FDPS	farnesyl diphosphate synthase	14435210	2,17	0,0004
EBP	emopamil binding protein (sterol isomerase)	14458384	2,03	0,0005
PRTFDC1	phosphoribosyl transferase domain containing 1	14319232	2,13	0,0008
OCLN	occludin	14320896	2,01	0,001
OMD	osteomodulin	14257861	2,3	0,0012
OLFM3	olfactomedin 3	14423212	2,13	0,0015
SEMA3D	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	14313894	2,32	0,0017
CEMIP	cell migration inducing protein, hyaluronan binding	14378163	2,11	0,002
LOC612207	insulin growth factor-like family member 3	14259320	2,02	0,002
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble	14399798	2,19	0,0044
RND1	Rho family GTPase 1	14363003	2,04	0,0048
CCL17	chemokine (C-C motif) ligand 17	14325178	2,21	0,005

Supplementary table S4: Downregulated genes in treated PC2 cells

Gene Symbol	Gene Name	Entrez ID	FC	p value
HAS2	hyaluronan synthase 2	14288059	-3,12	3,27E-05
TMEM26	transmembrane protein 26	14406114	-3,12	5,69E-05
CBLN4	cerebellin 4 precursor	14352298	-3,02	0,000066
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	14273152	-2,63	7,38E-05
LAMA4	laminin, alpha 4	14285468	-2,73	8,32E-05
TRIB2	tribbles pseudokinase 2	14303727	-2,71	8,54E-05
MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	14409820	-3,04	0,0001
SMIM3	small integral membrane protein 3	14407764	-2,69	0,0001
IL7R	interleukin 7 receptor	14408372	-2,57	0,0001
IL1R1	interleukin 1 receptor, type I	14272400	-2,77	0,0002
SEMA6D	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	14380344	-2,27	0,0002
PDGFR-A	platelet-derived growth factor receptor, alpha polypeptide	14286984	-2,24	0,0002
MMP1	matrix metallopeptidase 1	14409830	-3,57	0,0003
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	14260883	-2,49	0,0003
IL18RAP	interleukin 18 receptor accessory protein	14272354	-2,15	0,0003
SLPI	secretory leukocyte peptidase inhibitor	14351829	-2,01	0,0008
MIR33B	microRNA mir-33b	14411283	-2,19	0,0011
SLC6A2	solute carrier family 6 (neurotransmitter transporter), member 2	14325370	-2,09	0,0014

LIN7A	lin-7 homolog A (C. elegans)	14298069	-2,07	0,0014
ACTC1	actin, alpha, cardiac muscle 1	14381724	-2,49	0,0025
CLIC6	chloride intracellular channel 6	14384771	-2,11	0,0059
LOC607368	Ig lambda chain V-I region BL2	14358995	-2,26	0,0087

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Capítulo 3- Trabalho científico 2

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Rapamycin inhibits mTOR pathway in canine prostate cancer *in vitro*, a promising treatment option

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Abstract

Dog spontaneously develop prostate cancer (PC) with high frequency, metastatsis and is associated with poor prognosis and limited efficacious treatment. The use of “in vitro” models to study drug efficacy is an important tool, and there is a need for studies like that in veterinary oncology. In this study, we performed microarray gene expression analysis in normal canine prostate and prostatic carcinoma cell cultures, to search for differently expressed genes. Among the 6,270 differentially expressed genes revealed in transcriptome, 3,242 were upregulated and 3,028 were downregulated, and some were related to PI3K/AKT/mTOR pathway activation, which was confirmed after enrichment analysis. Among genes involved in this pathway, we found increased expression levels of *FKBP1A, FKBP1B, AKT1S1, PDK2, PIP5K1, PIP5KL1* in canine prostate cancer compared to normal prostate cell. Considering the central role of mTOR in carcinogenesis and previous results of our group, we treated two canine PC cells lines with rapamycin, a mTOR inhibitor, and evaluated gene and protein expression of mTOR, AKT and 4E-BP1, from mTOR pathway. therapy. . We treated two canine prostate carcinoma cells (PC1 and PC2) with rapamycin *in vitro* (6, 10 and 12 µM) at 24 hour and there was a dose dependent decrease in cell viability. Our results showed *mTOR* and 4*E-BP1* were downregulated after rapamycin treatment and *AKT* overexpression in both PC celllines, that could represent a possible acquisition of resistance to treatment. On the other hand, rapamycin decreased phosphorylated AKT protein expression in PC1 cell line, but increased phosprhorylated 4E-BP1 protein expression in PC2 cell line. We suggest that mTOR inhibition is a potential choice for the treatment of canine prostate cancer, which supports future prostate carcinoma clinical trials. But acquired resistance to treatment remains a challenge and precision medicine could be an approach to overcome this problem.

Key words: mTOR, rapamycin, canine prostate carcinoma, predictive factor

1. Introduction

Prostate cancer is the second most frequent cancer in men worldwide with 1,276,106 new cases, leading to 358,989 cases of deaths in 2018[1]. The estimated new prostate cancer cases in 2020 were 191,930 and33,330 estimated cases of death [2]. In dogs, the incidence of prostate carcinoma is lower than found in humans, but dogs are the only nonhuman species in which occurs spontaneous prostate carcinoma (PC) [3,4]. Due to some similarities between the species, the dog is considered as a model for human prostate, mainly in advanced cases and androgen receptor negative[5,6]. However, canine PC presents some particularities, such as more aggressive histological pattern, high aggressiveness and androgen independency at diagnosis [3,4].

Although there are similarities in the development of PC between both species, there are some differences that needs to be considered for comparing human and canine PC [7]. In humans, the usual PC presents positive androgen receptor expression and dependency to androgenic hormones. Even in humans, only a specific tumor group lack AR expression. Thus, antiandrogenic drugs are the first line treatment for the majority of human PC [8]. On the other hand, canine PC usually lacks AR expression at the diagnosis and there is no standard treatment to for it. Thus, studies evaluating canine PC must take into consideration and compare canine PC with a specific human PC subset independent to androgen [3].

In a previous publication from our research group, we evaluated copy number alterations in canine PC samples compared to normal samples and performed a cross validation with human PC from The Cancer Genome Atlas (TCGA) [3]. Among the pathways involved in both human and canine PC, we identified alteration mTOR pathway, including *AKT* and *ELF4* gains and loss of *PTEN*. Furthermore, we validated the alterations in mTOR pathway in an independent set of canine PC samples[9]. Demonstrating that mTOR pathway is important for both human and canine PC development.

PC is biologically heterogeneous, complex and need several signal pathways for initiation, progression and angiogenesis[10]. The phosphatidylinositol-3kinase (PI3K)/AKT/ mammalian target of rapamycin (mTOR) pathway is one of these pathways and has been implicated in prostate carcinogenesis and castration resistance [11]. mTOR, a serine/ threonine-specific-

kinase, can be divided into two functionally distinct complexes (mTORC1 and mTORC2) and acts as both downstream effector and a upstream regulator in PI3K pathway[12,13]. Thus, mTOR inhibitors, like rapamycin, with antineoplastic potency have been developed and studied in several human solid malignancies, such as breast cancer, metastatic pancreatic cancer, urinary bladder cancer and PC [14–17].

Rapamycin, the first anticancer targeting mTOR, is a bacterial macrolide produced by *Streptomyces hygroscopicus*, initially identified as an antifungal agent and currently approved as an immunosuppressive agent for transplant recipients[18,19]. Rapamycin forms a complex with FK506-binding protein (FKBP12) and interacts with mTOR inhibiting its function, with loss of phosphorylation of the eukaryotic translation initiation factor (4E-BP1) resulting in association with translation factor eIF4E and inhibit translation [20,21].

The use of rapamycin treatment in canine cell culture has been described in osteosarcoma and melanoma, with a dose -dependent decrease in the cell lines [22,23]. A study with dogs presenting confirmed appendicular osteosarcoma demonstrated that rapamycin can be safely administered to this specie at pharmacokinetic exposures[24]. However, the clinical anticancer effect of rapamycin in dogs with PC have not been demonstrated and its efficacy is not known.

Due the previous evidence that mTOR pathway was deregulated in both human and canine PC, and that there is a need to better understand “in vitro” models for anticancer drug studies, we performed global gene expression in normal and two canine PC cell lines, to evaluate the differently expressed pathways, and from that, comparing with our previous results[3,] we used rapamycin as a drug target for mTOR pathway. Our aim was to evaluate rapamycin antitumor effect *in vitro* and assess whether treatment would alter genes and proteins expression involved in PI3K/AKT/mTOR pathway regulation in two canine PC cell lines.

2. Materials and methods

2.1. Ethical approval

All procedures were approved by the Ethics Committee on Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science of the São

Paulo State University, Botucatu, SP, Brazil (Protocol: 0004/2017) and performed in accordance with the National and International Recommendations for the Care and Use of Animals (National Research Council) [25].

2.2. Primary canine PC cell culture

Two characterized primary canine prostate cancer cell cultures (PC1 and PC2) from two different canine PCs and one normal canine prostate (NP) were collected during necropsy and cultured, stablished and characterized previously[26]. The cells were cultured in complete medium with DMEM F12 (Lonza Inc., Allendale, NJ, USA) at 37 °C in 5% CO_2, supplemented with 10% inactivated fetal bovine serum (FBS, HYCLONE, Waltham, MA, USA) and 100 U/mL of penicillin G and 100 mg/mL of streptomycin (SIGMA, Portland, OR, USA).

2.3. Microarray

RNA was extracted, isolated and purified from the three cell lines (PC1, PC2 and NP), according to the manufacturer´s protocol (RNeasy mini kit, Qiagen, Hilden, Germany). Each PC sample was extracted in duplicate and normal prostate in triplicate. The RNA concentration and purity were evaluated using spectrophotometry NanoDrop™, ND-8000 (Thermo Scientific, Waltham, MA, USA). We used the Bioanalyzer 2100 and the Agilent RNA 6000 Nano Series kit (Agilent Technologies, Santa Clara, CA, USA) to assess RNA integrity.

We used GeneChip® Gene 1.0 ST Arrays (Affymetrix, CA, EUA) to generate a global gene expression profile. The labeled cDNA was hybridized and detected according to the manufacturer´s instructions. Then, the chips were scanned in the Scanner 3000 7G series (Affymetrix, Santa Clara, CA, EUA) and Affymetrix CEL files were downloaded and processed by the Applied Biosystem™ Transcriptome Analysis Console (TAC, Affymetrix) Software. The selection criteria to select differentially expressed genes were a P value <0.05 and 2.0-fold change cutoff. Hierarchical clustering was applied to identify groups of similarly expressed genes and the result was represented by a heatmap using the TAC software.

2.4. Enrichment analysis

The differentially expressed genes between NP and PCs were subject to an enrichment analysis using Enrichr software (https://amp.pharm.mssm.edu/Enrichr/). Enrichment of biological pathways supplied by Kyoto Encyclopedia of Genes and Genome (KEGG 2019, human) was performed using Enrichr software. The resulting pathways were ranked according to the lowest p value.

2.5. Rapamycin anti tumoral effect in canine PC

The MTT assay was used to evaluate the rapamycin effect in viability of canine prostate carcinoma cell lines (PC1 and PC2 cells) and calculate the half maximal inhibitory concentration (IC50) value. In brief, $1x10^4$ cells/well of PC1 and PC2 cells were seeded into 96-well culture plates in 0.1 ml of complete medium. After 24 hours, the complete medium was removed and the cells were incubated with different concentrations of rapamycin (R0395 Sigma-Aldrich) diluted in dimethyl sulfoxide (DMSO): 6 µM, 10 µM and 12 µM (12 wells per concentration). Positive control was the cells treated with 0.4% DMSO. After 24 hours of incubation at 37ºC at 5% CO2, the medium was removed and samples were incubated with new medium and MTT 0.5 mg/ml (Invitrogen™, M6494) for 4 hours. The medium was removed from all plates and 200 µL of DMSO (Hybri-Max™; Sigma) was added to solubilize the precipitate MTT salts. The absorbance corresponding to each sample was determined at 595 nm on a multiwall plate reader.

Percentage of cell viability was calculated using the following formula: $(A_{treatment} - A_{blank})/ (A_{DMSO} - A_{blank}) \times 100\%$ [27], where A = absorbance, DMSO = vehicle control, blank = no cells.

2.6. Transcript expression by real time quantitative (RT-qPCR)

We used RT-qPCR to detect the gene expression level of *AKT*, *mTOR* and *4E-BP1* in PC1 and PC2 cells before and after treatment with rapamycin for 24 hours and evaluate the effects of rapamycin on the molecules involved in PI3/AKT/mTOR pathway. PC1 and PC2 cells were seeded at 1×10^6 cells/well into 6-well plate and maintained at 37ºC with 5%CO2. When reached 70-80%confluence, cells were treated with IC50 dose of rapamycin for 24 hours.

We removed the medium with rapamycin and the cells were washed twice with Dulbecco´s phosphate-buffered saline (DPBS). RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer´s instructions. We assessed the RNA concentration and purity by using Nanodrop ™ ND-8000 spectrophotometer (ThermoScientific) and RNA integrity using Bioanalyzer RNA 6000 Nano Kit (Agilent).

To eliminate genomic DNA, the extracted RNA was treated with 1U of *Dnase I Amplification Grade* (Life Technologies) in 10X *Dnase I Reaction Buffer* and 25mM EDTA pH 8.0. cDNA synthesis was performed using 1µg of total RNA treated with DNAse I (Life Technologies, Rockville, MD, USA), 200 U of *SuperScript* III Reverse Transcriptase enzyme (Life Technologies), 4 µL of SuperScript First-Strand Buffer 5X, 1 µL of dNTP 10 mM each (Life Technologies), 1 µL of random hexamers (100 ng/ µL) (Life Technologies), 1 µL of Oligo-$(dT)_{18}$ (500 ng/ µL) (Life Technologies) and 1 µL of DTT 0.1 M (Life Technologies) in a final volume of 20 µL. Reverse transcription was carried out at 50ºC for 60 min and then inactivated at 70ºC for 15 min. Reference genes were *GAPDH, HPRT,RPL8, RPS5* and *RPS19.* Gene relative quantification was calculated by the $2^{-\Delta\Delta CT}$ method[28].

2.7. Western Blot

For the Western blot analysis, PC1 and PC2 were lysed in M-PER (Mammalian Protein Extraction reagent, Thermo scientific, Rockford, IL, USA) before and after treatment with rapamycin for 24 hours. Protein samples (15 µg) were electrophoresed on 5% stacking gel, 8% or 10% resolving gel. The proteins were transferred to 0.45nm pore sized nitrocellulose membranes (Amersham Biosciences), blocked in 5% bovine serum albumin (BSA) for 1 hour. After that, the membranes were incubated with primary antibodies: 1:1000 phosphorylated AKT (Thr 308, 9275, Cell Signaling), 1:1,000 phosphorylated mTOR (Ser 2448, 2971, Cell Signaling) (results not shown in this article, due to technical problems), 1:1,000 phosphorylated 4E-BP1 (T37/46, 236B4, Cell Signaling) overnight at 4ºC. Finally, the 1:10,000 diluted secondary antibodies (anti-rabbit) were incubated for 2 hours and target bands were detected using Amershan ECL Prime Western Blotting Detection Reagents (GE Healthcare). β-

actin (C4, Sc47778, Santa Cruz Biotechnology) was used as the loading control.

2.8. Statistical analysis

Protein expression levels of each antibody was quantified with ImageJ software (National Institute of Health). Transcript levels were analyzed by t-test. The statistical analysis, the IC50 determination and graphics were obtained with GraphPad Prism 8.0.2 (GraphPad Software, Inc., La Jolla, USA). P values lower than 0.05 were considered statistically significant.

3. Results

3.1. Microarray

There were 6270 differentially expressed genes (DEGs) ($p<0.05$) comparing normal and PC cell lines (PC1 and PC2), consisting of 3242 upregulated and 3028 downregulated DEGs (figure 1). Heatmap of DEGs between NP and PC cells were plotted (Supplementary Figure 1). When we compared PC1 and PC2 cell lines, it was observed 423 downregulates and 697 upregulated genes.

Comparing normal cells with both PC cell lines, the DEG list showed that out of 6,270 genes, 1,532 genes had neither gene name or symbol, comprised of 508 upregulated and 1,024 downregulated DEGs, resulting in 2,734 upregulated genes and 2,004 downregulated genes. We found upregulated DEGs related in PI3K-AKT-mTOR pathway, including *FKBP1A, FKBP1B, AKT1S1, PDK2, PIP5K1A, PIP5K*L1 and downregulated DEGs: *DEPTOR, FKBP2, PIP4K2A, PIP4k2C, PIP5k1B, PIP5K1C*.

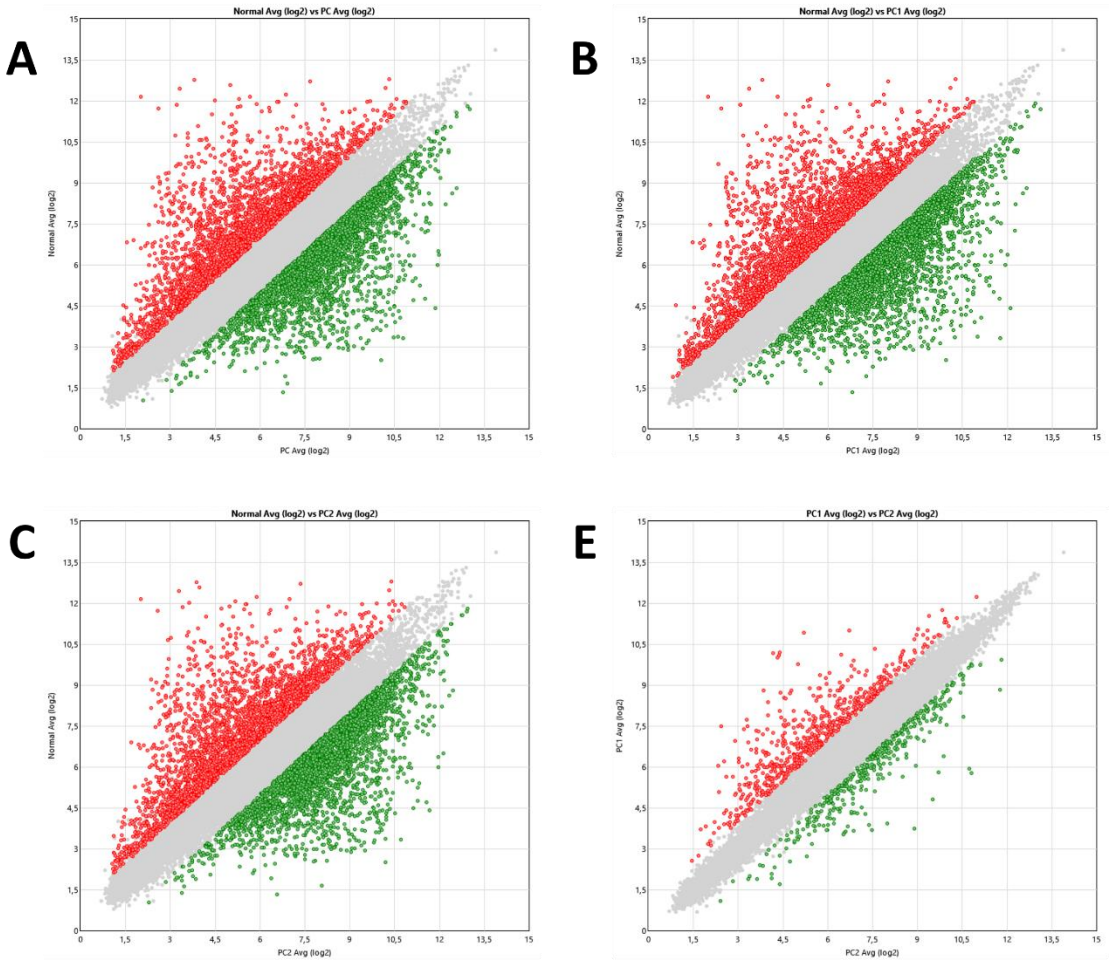

Figure 8. Gene expression analysis of canine normal and PC cell lines represented as a volcano plot. It is possible to observe a high number of downregulated (green dots) and upregulates (red dots) genes in normal cells compared to both PC cell lines A) and a proportional alteration when compared normal with PC1 (B) or PC2 (C). On the other hand, when compared PC1 and PC2 cell cultures (D), there is a lower number of deregulated genes.

3.2. Enrichment analysis

A total of 2,734 upregulated and 2,004 downregulated DEGs were used in the enrichment analysis. KEGG demonstrated upregulated (Supplementary Table 1) and downregulated (Supplementary Table 2) DEGs, related to PI3K/AKT pathway ($p<0.05$). However, this ranking included terms nonrelated to prostate carcinogenesis. When considering only the term “pathway”, PI3K signaling pathway was the 7th and 8th on the list of up and downregulated DEGs, respectively.

According to KEGG pathway, the up and downregulated genes associated with PI3K signaling pathway are described in table 1.

Table 1: Upregulated and downregulated genes between canine normal prostate and prostatic carcinoma cell lines, involved in PI3K signaling pathway.

Upregulated genes	*ITGB1, CDKN1A, CSF1, YWHAB, ITGB3, PI3KCD, LAMC2, BRCA1, PI3KCB, FGF5, GHR, GYS, CCD2, CREB3L3, PPP2R1B, YWHAQ, CREB3L1, MYC, ITGAV, RAC1, YWHAG, PDGFRA, MAP2K1, IL4R, MAP2K2, ITGA3, PDPK1, ITGA2, OSMIR, NGF, YWHAZ, EREG, CCNE2, CCNE1, CDC37, COL6A1, ITGA7, SGK3, ITGA6, ITGA5, SGK1, MET, EPHA2, TLR2, IFNAR1, PDGFB, PDGFA,EFNA5, THBS1, PPP2CA, NRAS, GNG2, GNG4, GNG7, THEM4, PKC2, ANGPT1, FN1, VEGFC, PTK2, COL1A1, COL1A2, G6PC3, CDK6, CDK4, ITGA10, CDK10, MDM2, KRAS, IL7RR, FGFR3, BCL2L1*
Downregulated genes	*CDKN1B, PRKAA2, ITGB5, ATF6B, LAMA2, ITGB4, LPAR1, TNC, PIK3R2, PIK3R1, FOXO3, FGF2, IGF1R, VTN, ERBB3, CREB3L4, ERBB4, PDGFD, CREB3L2, ERBB2, KDR, ITGB8, IL6R, NTRK2, ANGPT2, VWF, INSR, MAGI2, ITGA1, IGF1, GNG12, PRLR, EFNA1, NR4A1, PPP2R2C, IL7, PPP2R2B, GNB4, ITGA8, COL4A5, GNB3, TEK, PIK3AP1, TLR4, FGFR2, FGF10, ITGA9.*

3.3. Rapamycin anti tumoral effect in canine PC

After rapamycin treatment, a significant decrease in the number of viable cells was observed in PC1 and PC2 cells as compared to control. After 24 hours, in PC1 cells, rapamycin at concentrations 6, 10 and 12 µM reduced cell viability to 93.16%, 75.42% and 46.65%, respectively (figure 2A); and 95.57%, 62.36% and 36.87% in PC2 cells (figure 2B). The IC50 values of rapamycin were 11.47 µM (figure 2C) and 10.58 µM (figure 2D) for PC1 and PC2, respectively.

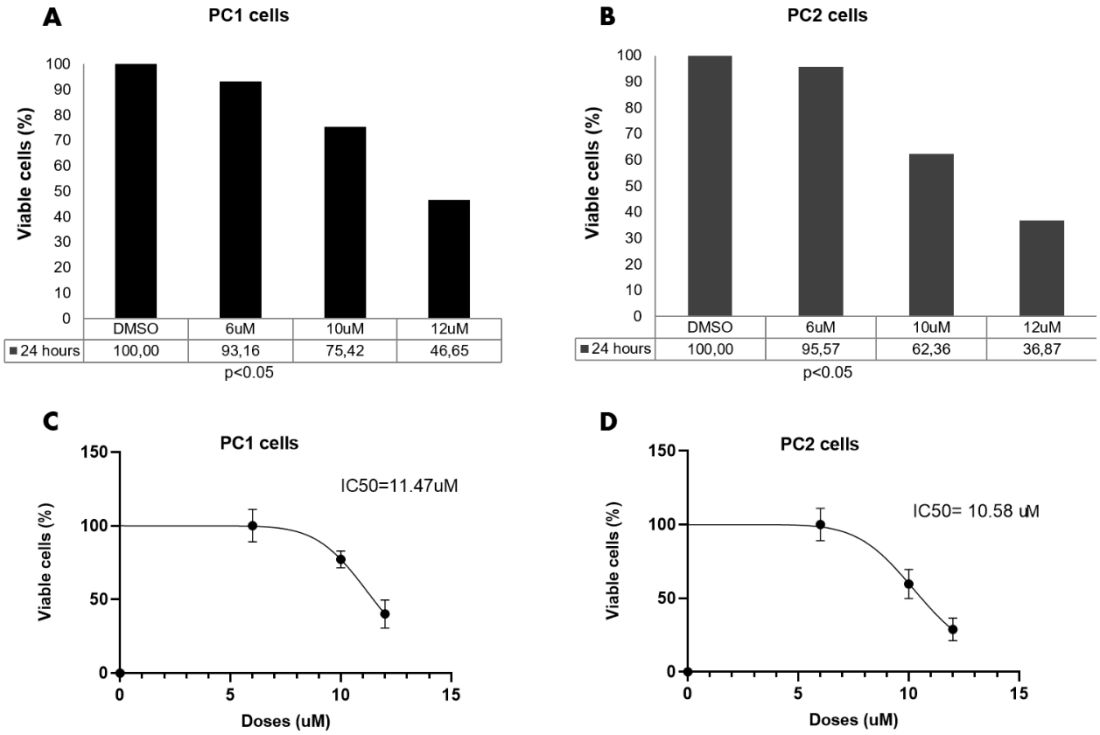

Figure 9: The effect of rapamycin on viability of canine prostate carcinoma cells by MTT assay at 24 hours. Three rapamycin concentrations (6 µM, 10µM, 12µM) were used. PC1 cells (A) and PC2 cells reduced significantly ($p<0.05$) in a dose-dependent manner comparing to control (DMSO). The IC50 of rapamycin was 11.47 µM and 10.58 µM in PC1 cells (C) and PC2 cells (D), respectively.

3.4. Transcript expression by real time quantitative (RT-qPCR)

Significant differences ($p<0.05$) were observed in *AKT, mTOR* and *4E-BP1* gene expression among the untreated and treated canine PC cells (figure 3), except *mTOR* in PC1 cells. Results from RT-qPCR analysis showed that PC1 (figure 3A) and PC2 (figure 3D) cells exposed to IC50 of rapamycin for 24 hours significantly upregulated *AKT* gene. The mean RQ value for *AKT* was 0.921 in non-treated PC1 cells and 1.362 in treated PC1 cells. In PC2 cells, the mean RQ was 1.000 and 1.311 in non-treated and treated, respectively.

mTOR transcript levels did not alter in untreated group comparing to treated PC1 cells (figure 3B) (mean= 0.792). However, treatment with rapamycin decreased mTOR transcript levels in PC2 cells (figure 3E) (mean= 0.744) comparing to untreated group (mean = 1.002).

We observed low 4E-BP1 transcript levels in treated PC1 (figure 3C) (mean= 0.418) and PC2 (figure 3F) (mean = 0.538) cells compared to non-treated PC1 and PC2 cells, respectively.

3.5. Western Blot

We investigated the effect of rapamycin treatment on p-AKT, p-mTOR and p-4E-BP1 expression. Western blot showed reduced p-AKT in PC1 treated cells (figure 3G), as well an increase p-4E-BP1 levels (figure 3H) in PC2 treated cells.

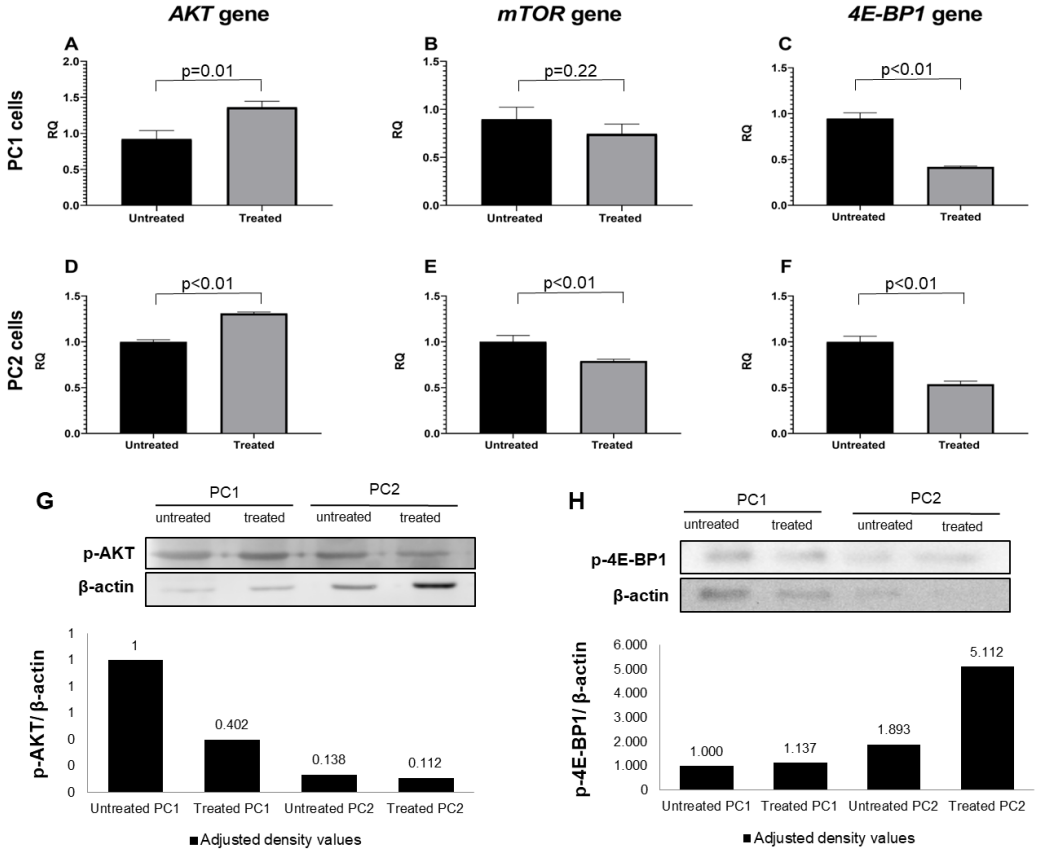

Figure 10: Relative quantification (RQ) values of *AKT* (A), *mTOR* (B) and *4E-BP1* (C) in untreated and treated PC1 cells. The RQ of the same genes observed in untreated and treated PC2 cells (D, E, F) with IC50 dose of rapamycin for 24 hours. Western blot analysis of p-AKT (G) and p-4E-BP1 (H) protein levels in untreated and treated PC cells (PC1 and PC2).

4. Discussion

In the present study, we used microarray to explore variations between normal and canine prostate carcinoma cell lines, developed by our group. Results like these are important for veterinary and comparative oncology since they lack in the literature, and can be used for future predictive comparative studies.

. We found 6,270 DEGs and some of them were genes related to PI3K/AKT/mTOR pathway, which plays an important role in controlling cell

growth and proliferation, while their deregulation results in pathological conditions, as carcinogenesis of different cancers, including prostate carcinoma [11,29,30]. In previous work, we revealed alterations in proteins related to this pathway, including an increase of p-mTOR and eIF4E in higher Gleason canine prostate cancer score values and metastasis, respectively [9], as well as gains in *AKT* and *ELF4*, from mTOR pathway in canine PC, by aCGH[3]. Besides, a decrease of PTEN (phosphatase and tensin homolog) was observed in canine prostate carcinoma compared to normal samples[31], which negatively regulates downstream PI3K family members, including mTOR[32]. mTOR pathway is important in human and canine PC[9,33] , adding novel information regarding the use of the dog as a natural model for comparative oncology and translational research.

In canine PC cell lines, we found that the *PIP5K1A* (PI-4-phosphatase 5 kinase α) and *PIP5KL1* (PI-4-phosphatase 5-kinase-like 1) genes were upregulated, which generates PIP2 from PIP4 (PI-4- phosphatase) [34], contributing to increase of PI3K/AKT/mTOR activation. The pathway can be activated by upstream signaling of a variety of molecules and phosphorylation of PIP2 generates PIP3 that leads to AKT phosphorylation [34,35]. After PIP2 phosphorylation to generate PIP3, AKT activity depends on phosphorylation of two sites: the threonine 308 (AKT T308) and serine 473 (AKT S473)[36], and one of upregulated DEG found in PC was *PDK2* (*S473 kinase*) which is one of kinases able to phosphorylate AKT S473, showing an possible mechanism for mTOR pathway upregulation, as previous demonstrated in canine PC, by copy number variation and protein expression[3,9].

We found increase of *FKBP1A* (*FKBP12*) and *FKBP1B* (*FKBP12.6*) in the PC cell lines and both are rapamycin and FK506[37] receptor . The FKBP12-rapamycin-mTOR restricts the access of mTOR substrates and destabilizes mTORC1 (mechanistic target of rapamycin complex 1) integrity, inhibiting PI3K/AKT/mTOR pathway [38]. Therefore, the presence of *FKBP1A* and *FKBP1B* represent important genes to act as a PI3K/AKT/mTOR suppressor when rapamycin treatment is considered.

In addition, mTORC1 also contains tumor suppressors genes, including *DEPTOR* (DEP-domain containing mTOR-interacting protein) which is a naturally negative regulator of mTOR[39,40]. Our results demonstrated

downregulation of *DEPTOR* in canine PC cell lines, also contributing to mTOR signaling. *DEPTOR* expression was decreased in human prostate cancer and its role in prostate tumorigenesis was confirmed with *DEPTOR* -knockout, suggesting the potential use of mTOR inhibitors to treat PC with low DEPTOR expression [41].

Taking together the altered gene expression in canine PC cell lines, our previous results [3,9], the lack of an effective treatment for canine PC and the enrichment analysis done, that revealed DEGs involved in PI3K/AKT pathway and one of the top 10 pathways in up and downregulated DEGs, we treated our canine PC cell lines with an mTOR inhibitor, rapamycin, a drug tolerated by dogs, that was already used with good results in canine osteosarcoma[42] and melanoma[23].

Rapamycin significantly ($p<0.05$) decreased the viability of both PC1 and PC2 cell lines in a dose-dependent manner, showing that canine prostate carcinoma cells were sensitive to treatment. We found increase of *AKT* genes in both treated PC cells, remaining a possible mechanism of tumor drug resistance. Activation of AKT by mTOR inhibition was reported in several types of cancer including lung [43], breast and prostate cancer cell lines [44]. However, we observed decrease phosphorylated AKT protein expression. The decrease in p-AKT after rapamycin treatment has been demonstrated in acute myeloid leukemia cells via suppression of mTORC2[45]. This complex 2 was thought to be rapamycin insensitive, however prolonged treatment (24 hours) inhibits the assembly of mTORC2 and decrease the levels of mTORC2 in some cells [46,47].Taken together, these evidences support the therapeutic potential of mTOR inhibitor.

Rapamycin mechanism of action is to bind the cytosolic protein FK506 binding protein 12 (FKBP12), inhibiting mTOR phosphorylation, a reversible process, by binding to FRB domain [48,49]. As described before, rapamycin does not affect transcript levels of mTOR directly. Interestingly, we observed *mTOR* gene downregulated in treated PC2 cells ($p<0.05$) and a tendency to decrease in PC1 cells. One supposed reason for the result is that mTOR activity can be affected by direct, indirect mechanisms, and a complex of upstream signaling elements, modulating a downstream signaling intimately linked with feedback loops connecting mTOR transcription [13,50–52]. Besides, transcription factors as

increasing NRF2 (NF-E2-related factor), responsible for the activation of cellular antioxidant response, results in transcriptional activation of *mTOR* gene [53].

Another effect of rapamycin treatment in canine PC cells was the decrease in *4E-BP1* transcript levels. Although we observed responsiveness of PC2 cells to treatment, the PC2 cell had an increase p-4E-BP1 after the treatment. Phosphorylation of 4E-BP1 has been correlated with cancer resistance to mTOR inhibitor therapy [54]. However, there are several candidates and feedback loops to be responsible for 4E-BP1 phosphorylation, decreasing its association with eIF4E and promoting translation protein[55]. Taking into consideration the binding between these proteins, the activation of 4E-BP1 and determination of cellular response to rapamycin can be performed using the ratio between 4E-BP1: eIF4E[56,57]. Therefore, there are still controversial results related with p-4E-BP1, for example in gastric cancer, that high protein expression was associated with favorable prognostic[58].

As the result of this study, we identified several differentially expressed genes between canine PC and normal prostate cell cultures. These DEGs are participants of several process and pathways, including PI3K/AKT/mTOR. Notably, it was revealed that rapamycin can decrease canine PC cell lines viability and may be considered as a treatment option for dogs with prostate cancer However, some gene alterations after rapamycin treatment may lead to drug resistance that is clearly an important emerging problem. The data support previous findings on the importance of PI3K/AKT/mTOR in canine prostate carcinoma, and consider that rapamycin treatment can benefit cancer patients in personalized therapy.

5. References

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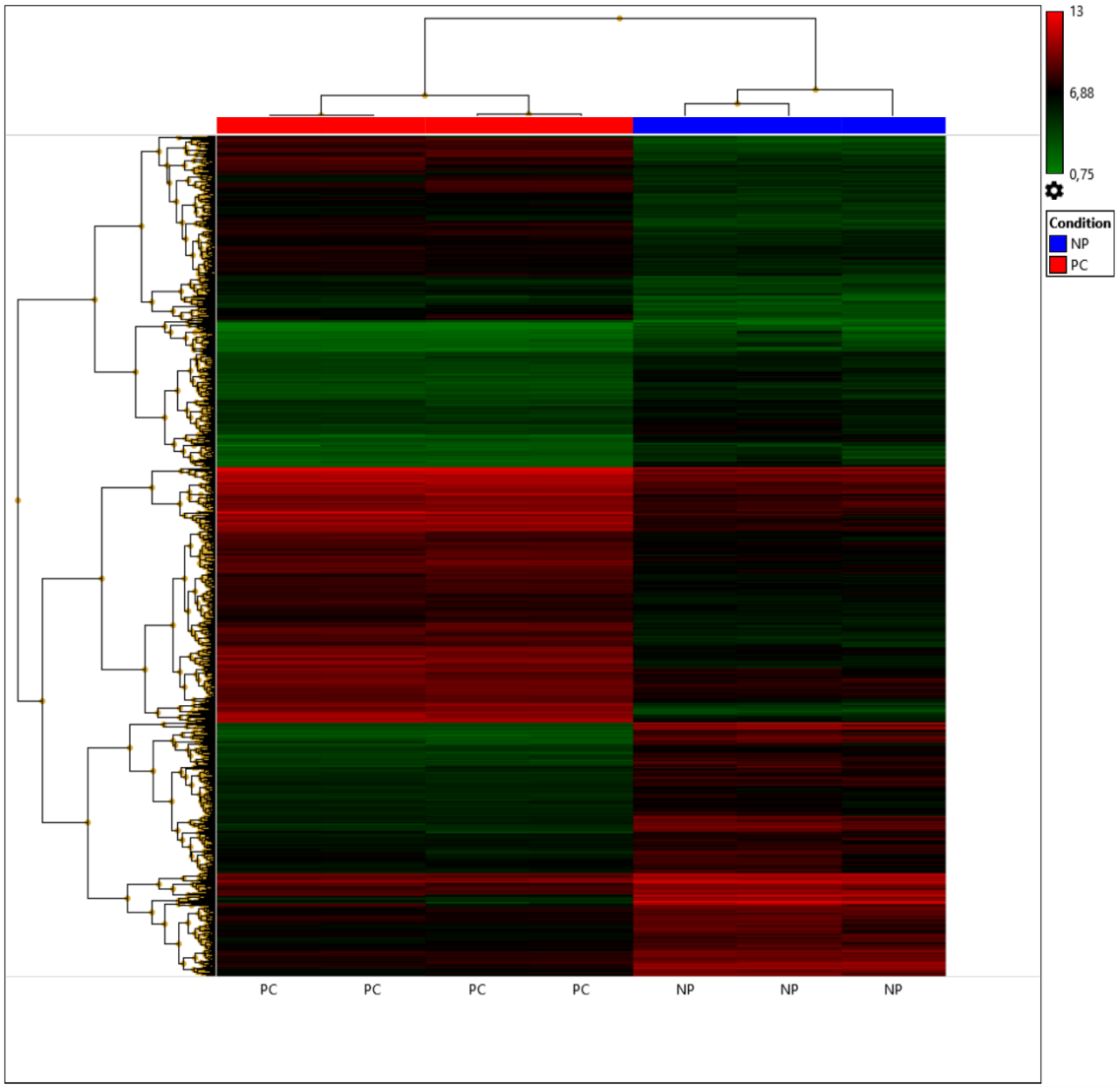
Supplementary files

Supplementary Figure 1: Hierarchical clustering heatmap for differentially expressed genes between PC (prostate cancer) and NP (normal prostate) of dogs. Columns correspond to samples and rows correspond to genes. The differences are highlighted in red (upregulated genes) and green (downregulated genes). Red color indicates upregulated DEGs and green color indicates downregulated DEGs.



Supplementary Table 1: Top 100 enriched pathways listed according to p value of differentially expressed genes upregulated in canine prostate cancer compared to normal prostate.

Index	Name	P-value
1	Cell cycle	1,05E-24
2	DNA replication	2,06E-11
3	Proteasome	3,17E-08
4	Cellular senescence	4,28E-08
5	Bladder cancer	8,59E-06
6	Human T-cell leukemia virus 1 infection	8,45E-05
7	Proteoglycans in cancer	1,31E-04
8	Mismatch repair	1,55E-04
9	Epstein-Barr virus infection	3,23E-04

10	Progesterone-mediated oocyte maturation	3,43E-04
11	Ribosome biogenesis in eukaryotes	5,78E-04
12	Oocyte meiosis	6,63E-04
13	p53 signaling pathway	0.000001049
14	Regulation of actin cytoskeleton	0.000002664
15	Fanconi anemia pathway	0.000002854
16	Hepatitis B	0.000003353
17	Pathways in cancer	0.000005298
18	Shigellosis	0.000006440
19	FoxO signaling pathway	0.000007714
20	Viral carcinogenesis	0.000009404
21	RNA transport	0.00001133
22	Bacterial invasion of epithelial cells	0.00002220
23	Axon guidance	0.00005673
24	Small cell lung cancer	0.00006447
25	Sphingolipid signaling pathway	0.00007478
26	Central carbon metabolism in cancer	0.00007564
27	Glioma	0.00008736
28	AGE-RAGE signaling pathway in diabetic complications	0.00009613
29	Colorectal cancer	0.0001158
30	Glycolysis / Gluconeogenesis	0.0001563
31	Human immunodeficiency virus 1 infection	0.0001745
32	Renal cell carcinoma	0.0001964
33	PI3K-Akt signaling pathway	0.0001965
34	Pathogenic Escherichia coli infection	0.0002012
35	Fc gamma R-mediated phagocytosis	0.0003068
36	Chronic myeloid leukemia	0.0003104
37	Prostate cancer	0.0003586
38	Purine metabolism	0.0003747
39	Melanoma	0.0003763
40	Endocytosis	0.0004627
41	Ubiquitin mediated proteolysis	0.0005402
42	Apoptosis	0.0005776
43	One carbon pool by folate	0.0005939
44	Thyroid hormone signaling pathway	0.0006008
45	Homologous recombination	0.0006203
46	Rap1 signaling pathway	0.0006232
47	TGF-beta signaling pathway	0.0006435
48	Pancreatic cancer	0.0006859
49	Non-small cell lung cancer	0.0008377
50	Kaposi sarcoma-associated herpesvirus infection	0.0008786
51	Human papillomavirus infection	0.0009460
52	Hippo signaling pathway	0.001194
53	Citrate cycle (TCA cycle)	0.001203
54	Hepatitis C	0.001296
55	Alanine, aspartate and glutamate metabolism	0.001438
56	VEGF signaling pathway	0.001525
57	Focal adhesion	0.001873
58	Neurotrophin signaling pathway	0.001990
59	Measles	0.002581
60	Nucleotide excision repair	0.002749
61	Pyrimidine metabolism	0.002782
62	Fluid shear stress and atherosclerosis	0.002902
63	Hepatocellular carcinoma	0.002905
64	Epithelial cell signaling in Helicobacter pylori infection	0.003176
65	RNA degradation	0.003405
66	ErbB signaling pathway	0.003739
67	MicroRNAs in cancer	0.003955
68	Pyruvate metabolism	0.004028
69	Pentose phosphate pathway	0.004455
70	Nitrogen metabolism	0.004507

71	ECM-receptor interaction	0.005361
72	TNF signaling pathway	0.005433
73	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.006103
74	HIF-1 signaling pathway	0.006412
75	Human cytomegalovirus infection	0.006571
76	Relaxin signaling pathway	0.007407
77	Fc epsilon RI signaling pathway	0.007572
78	Sphingolipid metabolism	0.007652
79	C-type lectin receptor signaling pathway	0.01042
80	Spliceosome	0.01121
81	Choline metabolism in cancer	0.01139
82	Adherens junction	0.01355
83	Glutathione metabolism	0.01458
84	Hypertrophic cardiomyopathy (HCM)	0.01679
85	Ras signaling pathway	0.01773
86	Galactose metabolism	0.01796
87	Terpenoid backbone biosynthesis	0.02155
88	Biosynthesis of unsaturated fatty acids	0.02222
89	Gap junction	0.02375
90	Cushing syndrome	0.02485
91	NF-kappa B signaling pathway	0.02674
92	Base excision repair	0.02683
93	Dilated cardiomyopathy (DCM)	0.03273
94	MAPK signaling pathway	0.03543
95	Phospholipase D signaling pathway	0.03823
96	Gastric cancer	0.04124
97	Platelet activation	0.04129
98	Inflammatory mediator regulation of TRP channels	0.04364
99	Glycosaminoglycan biosynthesis	0.04641
100	Melanogenesis	0.04778

Supplementary Table 2: Top 100 enriched pathways listed according to p value of differentially expressed genes downregulated in canine prostate cancer compared to normal prostate.

Index	Name	P-value
1	Dilated cardiomyopathy (DCM)	0.000001047
2	Lysosome	0.00001333
3	Rap1 signaling pathway	0.00001401
4	Tight junction	0.00003193
5	Hypertrophic cardiomyopathy (HCM)	0.00004329
6	cGMP-PKG signaling pathway	0.0001121
7	Inositol phosphate metabolism	0.0001841
8	Axon guidance	0.0002636
9	Morphine addiction	0.0003587
10	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.0004016
11	Insulin secretion	0.0004649
12	Valine, leucine and isoleucine degradation	0.0004657
13	Regulation of lipolysis in adipocytes	0.0005693
14	Adrenergic signaling in cardiomyocytes	0.0006530
15	Leukocyte transendothelial migration	0.0009585
16	Serotonergic synapse	0.001084
17	Parathyroid hormone synthesis, secretion and action	0.001104
18	Phosphatidylinositol signaling system	0.001114

19	cAMP signaling pathway	0.002223
20	MAPK signaling pathway	0.002450
21	Vascular smooth muscle contraction	0.003822
22	Thyroid hormone synthesis	0.004282
23	Glucagon signaling pathway	0.004304
24	Histidine metabolism	0.004740
25	Circadian entrainment	0.005031
26	Endocrine and other factor-regulated calcium reabsorption	0.005206
27	Salivary secretion	0.005241
28	Aldosterone synthesis and secretion	0.005620
29	Renin secretion	0.005628
30	Cortisol synthesis and secretion	0.008462
31	Propanoate metabolism	0.009509
32	GABAergic synapse	0.01069
33	Proteoglycans in cancer	0.01084
34	AMPK signaling pathway	0.01090
35	Prostate cancer	0.01115
36	Relaxin signaling pathway	0.01303
37	Regulation of actin cytoskeleton	0.01460
38	Insulin resistance	0.01518
39	Platelet activation	0.01537
40	PI3K-Akt signaling pathway	0.01564
41	Tryptophan metabolism	0.01707
42	Longevity regulating pathway	0.01794
43	Cholinergic synapse	0.02144
44	Glyoxylate and dicarboxylate metabolism	0.02169
45	Estrogen signaling pathway	0.02254
46	Aldosterone-regulated sodium reabsorption	0.02269
47	Fatty acid degradation	0.02281
48	Vasopressin-regulated water reabsorption	0.02281
49	Purine metabolism	0.02287
50	Calcium signaling pathway	0.02379
51	Lysine degradation	0.02428
52	ECM-receptor interaction	0.02474
53	Glutamatergic synapse	0.02523
54	Dopaminergic synapse	0.02655
55	Gastric acid secretion	0.02660
56	Long-term depression	0.02723
57	Thyroid hormone signaling pathway	0.02951
58	Oxytocin signaling pathway	0.03791
59	Cell adhesion molecules (CAMs)	0.03911
60	Ras signaling pathway	0.03986
61	Apelin signaling pathway	0.04029
62	Insulin signaling pathway	0.04029
63	SNARE interactions in vesicular transport	0.04094
64	Cocaine addiction	0.04293

65	Focal adhesion	0.04456
66	Retrograde endocannabinoid signaling	0.04720
67	Cholesterol metabolism	0.04804
68	Pancreatic secretion	0.04891
69	Mineral absorption	0.05354
70	ABC transporters	0.06417
71	Renin-angiotensin system	0.06471
72	Circadian rhythm	0.07289
73	beta-Alanine metabolism	0.07289
74	Signaling pathways regulating pluripotency of stem cells	0.07682
75	Arachidonic acid metabolism	0.07900
76	Gap junction	0.07979
77	Adherens junction	0.08421
78	Bile secretion	0.08421
79	FoxO signaling pathway	0.08477
80	Glycine, serine and threonine metabolism	0.08614
81	Progesterone-mediated oocyte maturation	0.09424
82	Ovarian steroidogenesis	0.09596
83	Non-small cell lung cancer	0.1009
84	Peroxisome	0.1017
85	Steroid biosynthesis	0.1043
86	Autophagy	0.1103
87	Collecting duct acid secretion	0.1133
88	Butanoate metabolism	0.1275
89	Glycosphingolipid biosynthesis	0.1392
90	Type II diabetes mellitus	0.1513
91	Melanoma	0.1540
92	Pentose phosphate pathway	0.1583
93	Fc gamma R-mediated phagocytosis	0.1661
94	Rheumatoid arthritis	0.1661
95	HIF-1 signaling pathway	0.1661
96	Inflammatory mediator regulation of TRP channels	0.1661
97	Glutathione metabolism	0.1692
98	Pyruvate metabolism	0.1700
99	Breast cancer	0.1758
100	Proximal tubule bicarbonate reclamation	0.1766

Capítulo 4

DISCUSSÃO GERAL

Devido a importância do cão como modelo animal para estudos na oncologia comparada associado a importância do carcinoma prostático nas espécies canina e humana, além de ausência de terapias efetivas principalmente em casos avançados e metastáticos, faz- se necessário o estudo de novas terapias no tratamento do carcinoma prostático.

O presente trabalho objetivou realizar o tratamento *in vitro* de células de carcinoma prostático canino com o fosfato de toceranib e a rapamicina, avaliar o efeito na viabilidade celular, além de verificar o perfil de expressão gênica e proteica associadas ao tratamento estabelecido.

Observou-se que apesar das culturas de células prostáticas caninas distintas apresentarem o mesmo padrão de expressão proteica de proteínas alvo do receptor tirosina quinase (VEGFR2, PDGFR-β e c-KIT), a resposta à terapia foi diferente. Bem como a utilização do fosfato de toceranib interferiu nas células neoplásicas de forma diferente na expressão gênica após tratamento. Dentre os genes alterados após tratamento estão presentes genes relacionados com a via PDGFR, mas também relacionados com resistência terapêutica. Esses resultados corroboram para a importância da heterogeneidade do tumor, além dos mecanismos de resistência que devem ser considerados nas terapias antineoplásicas.

O presente estudo também comparou a cultura de células epiteliais advindas de próstata normal com duas culturas distintas de células neoplásicas de carcinoma prostático canino, e observou alterações de genes relacionados com a via PI3K/AKT/mTOR, sugerindo a possibilidade do uso da rapamicina nos carcinomas prostáticos caninos. A avaliação do efeito na viabilidade celular após tratamento com rapamicina mostrou diminuição de maneira dose depende das células de cultura celular provindas de dois carcinomas prostáticos caninos distintos. Porém, as alterações gênicas e proteicas de AKT, 4E-BP1 e mTOR sugerem a presença de possíveis mecanismos de resistência após o tratamento.

CONCLUSÕES GERAIS

Diante do exposto, as utilizações do fosfato de toceranib e da rapamicina apresentam certa resposta biológica *in vitro*, porém necessitam maiores pesquisas para que se obtenham maiores informações sobre a eficácia dos tratamentos em carcinomas prostáticos caninos. Além disso, devido a presença de possíveis mecanismos de resistência em ambos os tratamentos, deve-se considerar a medicina personalizada ou de precisão para obter-se uma melhor resposta antineoplásica.

As células de carcinoma prostático canino apresentaram perfil de expressão gênica e resposta antitumoral distintas, indicando uma heterogeneidade intertumoral. A célula PC2 apresentou uma maior resistência ao fosfato de toceranib, e pode ser utilizada como modelo de resistência quimioterápica intrínseca dos carcinomas prostáticos caninos.

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