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MicroRNA-101 as a potential regulator of mTORC2 and HIF-2α in Renal Cell Carcinoma

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Science is not only a disciple of reason but, also, one of romance and passion.

Stephen Hawking

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Abbreviations

A

ACTB – Beta Actin

AKT – Protein Kinase B

ATM – Ataxia Telangiectasia mutated

C

c-Met – Hepatocyte growth factor receptor

ccRCC – clear cell Renal Cell Carcinoma

chRCC – chromophobe Renal Cell Carcinoma

D

DEPTOR – Dep-domain-containing mTOR-interacting protein

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

DNA-PKcs – DNA-dependent protein kinase catalytic subunit

E

EGF – Epidermal Growth Factor

F

FBS – Fetal Bovine Serum

FKBP – FK binding Protein

G

GAP – GTPase-activating protein

GUSB – Beta Glucoronidase

H

HIF – Hypoxia-inducible factor

I

IFN-α - Interferon alpha

IL-2 – Interleukin 2

IL-8 – Interleukin 8

ITS – Insulin-transferrine selenium

M

MAPK – Mitogen-activated Protein Kinase

miRNA – MicroRNA

mLST8 – mammalian Lethal with Sec 13 protein 8

mRNA – messenger ribonucleic acid

mSIN1 – mammalian Stress-activated protein kinase interacting protein

mTOR – mechanistic target of rapamycin

P

PCR – Polymerase Chain Reaction

PD-1 – Programmed death 1

PD-L1 – Programmed death ligand 1

PDGF – Platelet-derived Growth Factor

PDK1 – Phosphoinositide-dependent kinase 1

PI3K – Phosphoinositide 3-kinase

PRAS40 – Proline-rich AKT substrate 40 KDa

pRCC – papillary Renal Cell Carcinoma

Pri-miRNA – Primary miRNA

Protor-1 – Protein observed with Rictor-1

PTEN – Protein phosphatase and tensin homolog

R

Raptor – Regulatory-associated protein of mTOR

RCC – Renal Cell Carcinoma

Rictor – Rapamycin-insensitive companion of mTOR

RISC – RNA-induced silencing complex

RORENO – Registo Oncológico Regional do Norte

RPMI – Roswell Parl Memorial Institute

T

TKI – Tyrosine Kinase inhibitor

TSC1/TSC2 – Tuberous Sclerosis complex

U

UTR – Untranslated Region

V

VEGF – Vascular Endothelial Growth Factor

VEGFR – Vascular Endothelial Growth Factor Receptor

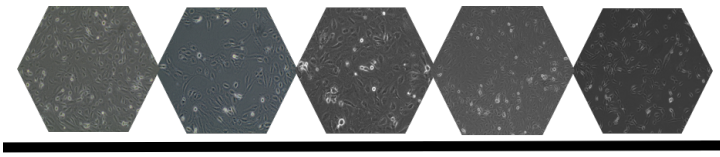
VHL – von Hippel-Lindau

W

WHO – World Health Organization

X

XPO5 – Nuclear Export Protein



Resumo

Resumo

O carcinoma de células renais (CCR) é o cancro sólido mais comum do rim no adulto, sendo o subtipo mais agressivo o carcinoma de células renais de células claras (CCRcc), existindo atualmente várias opções terapêuticas. Contudo, o CCR metastático permanece incurável, principalmente devido à aquisição de resistências ao tratamento. Além disso, aproximadamente 30% dos doentes já apresentam doença metastática no momento do diagnóstico, e 20 a 40% dos doentes com CCR localizado irão apresentar progressão da doença.

Uma das vias de sinalização envolvida na fisiopatologia do CCRcc é a via de *von Hippel-Lindau* (VHL). No entanto, a desregulação da via PI3K/AKT/mTOR também está envolvida na oncogénese deste cancro, sendo uma via central na regulação do metabolismo, proliferação e sobrevivência celular. Uma das abordagens terapêuticas usadas nos doentes de pior prognóstico são os inibidores do mTOR. Estas moléculas formam um complexo com a proteína de ligação FK e inibem a ligação do mTOR ao complexo 1 (mTORC1), inibindo assim os seus efeitos a jusante. No entanto, o outro complexo, mTORC2, não é inibido. Assim, um possível mecanismo de resistência a esta terapia poderá ser consequência da ativação compensatória da via PI3K/AKT, provavelmente devido à sobre-regulação do mTORC2.

Atualmente admite-se que os microRNAs (miRNAs) estão também envolvidos na aquisição de resistências a terapias dirigidas em vários tipos de cancro. Os miRNAs são uma família de pequenos RNAs não codificantes responsáveis pela regulação génica a um nível pós-transcripcional e a sua desregulação está descrita em diversos tipos de cancro, incluindo o CCRcc. Assim, o estudo dos perfis de expressão de miRNAs durante o desenvolvimento de CCRcc e a sua influência na aquisição de fenótipos de resistência, será um potencial alvo de estudo.

O principal objetivo deste estudo foi clarificar a influência do miRNA-101 no desenvolvimento de resistência aos inibidores mTOR em CCRcc e o seu potencial como biomarcador molecular.

Para o desenvolvimento deste estudo foram usadas quatro linhas celulares como modelo *in vitro*: uma linha celular renal epitelial proximal tubular normal (HKC-8), duas linhas celulares de adenocarcinoma renal (786-O e FG-2) e uma linha celular renal tumoral resistente a everolimus (estabelecida durante o desenvolvimento deste projeto).

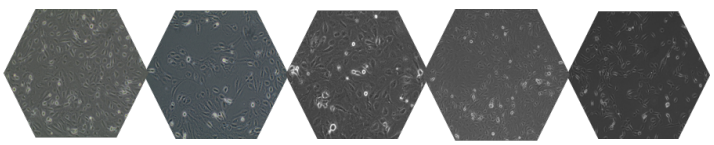
A quantificação dos níveis de miRNA-101 foi avaliada nas células (intracelular) e no respetivo meio de cultura (extracelular), a par da adicional quantificação relativa da expressão de mRNA do mTOR, Rictor, Raptor e HIF-2α.

De acordo com os nossos resultados, foi observado uma diminuição significativa dos níveis intracelulares de miRNA-101 nas duas linhas celulares tumorais, quando comparada com a HKC-8 (786-O *vs* HKC-8: P=0.030 e FG-2 *vs* HKC-8: P=0.003). Para além disso, este miRNA apresentou níveis extracelulares significativamente aumentados na linha celular FG-2 (P<0.001) e tendencialmente superior na 786-O (P=0.052). Adicionalmente, a expressão dos níveis intracelulares do miRNA-101 foi tendencialmente menor na linha celular resistente (786-OR) quando comparada com a linha celular 786-O (P=0.064). Foi também observado um aumento da expressão de miRNA-101 no meio das 786-OR (786-OR *vs* 786-O: P=0.004). Assim, ao longo da aquisição de resistência ao everolimus, as células começam a excretar maior quantidade de miRNA-101.

Em relação aos níveis de Rictor, observámos um aumento significativo na linha 786-O (P<0.001) quando comparado com a linha HKC-8. No entanto, observámos uma diminuição deste mRNA na linha FG-2 (FG-2 *vs* HKC-8: P=0.031). A sua expressão nas 786-OR é significativamente menor quando comparada com as 786-O (P=0.003). Relativamente ao Raptor, a expressão está aumentada em ambas as linhas tumorais comparativamente com as HKC-8 (786-O *vs* HKC-8: P<0.001 e FG-2 *vs* HKC8: P=0.08) e significativamente menor nas 786-OR quando comparadas com as 786-O (P<0.001). Em relação ao HIF-2α, a sua expressão é significativamente maior em ambas as linhas tumorais comparativamente com as HKC-8 (786-O *vs* HKC-8: P<0.001 e FG-2 *vs* HKC-8: P=0.016) e nas 786-OR comparativamente com as 786-O (P=0.003).

De acordo com os nossos resultados, tanto o mTORC1 como o mTORC2 aparentam ser influenciados pelo everolimus, mas não completamente inibidos, o que poderá estar associado com o desenvolvimento de resistência. Outro principal fator neste fenótipo de resistência poderá ser a elevação dos níveis de HIF-2α.

Em conclusão, o miRNA-101 é um potencial biomarcador preditivo de resistência aos inibidores mTOR, uma vez que é excretado pelas células resistentes a estes. No futuro, a análise dos níveis circulantes de miRNA-101 em amostras sanguíneas de doentes com CCRcc poderá permitir uma melhor monitorização da resposta a este tipo de tratamento.



Abstract

Abstract

Renal cell carcinoma (RCC) is the most common solid cancer of the adult kidney, being the most aggressive subtype the clear cell RCC (ccRCC) and there are a wide variety of treatments. The metastatic disease remains incurable mainly in consequence of the acquisition of treatment resistances. Moreover, approximately 30% of patients' present with already metastasized disease at the time of diagnosis, and 20-40% of the patients with localized RCC will present disease progression.

One of the signaling pathways involved in the pathophysiology of ccRCC is the *von Hippel-Lindau* (VHL) pathway, however the deregulation of the PI3K/AKT/mTOR pathway is also involved in the oncogenic mechanisms observed in ccRCC, and it serves as a central regulator of cell metabolism, proliferation and survival. One therapeutic approach used in ccRCC patients, that present a worst prognosis, are the mTOR inhibitors. These molecules form a complex with the FK binding protein and inhibit mTOR from binding complex 1 (mTORC1), thereby inhibiting its downstream effects. However, the other mTOR complex - mTORC2 - is not inhibited by these targeted therapies. Thus, the resistance mechanism can be mainly through compensatory activation of PI3K/AKT pathway probably via upregulation of mTORC2.

Additionally, microRNAs (miRNAs) also seem to be involved in targeted therapy resistance acquisition in several cancers. MiRNAs are a family of small non-coding RNAs, that are responsible for the regulation of gene expression at a post-transcriptional level, and have been reported as deregulated in several cancers including ccRCC. As so, it would be important to study the expression patterns of these molecules in ccRCC and evaluate their influence in resistance acquisition phenotypes, specially their influence in mTOR inhibitors patients' response.

The main aim of this study was to clarify the influence of miRNA-101 in ccRCC resistance development to mTOR inhibitors and its potential as molecular biomarker. To perform this study, four cell lines were used as *in vitro* model: one normal renal proximal epithelial tubular cell line (HKC-8), two renal adenocarcinoma cell lines (786-O and FG-2) and an everolimus-resistant tumoral renal cell line (786-OR), the last one established during the development of this project. The quantification of miRNA-101 expression in the cells (intracellular) and in the respective medium (extracellular) was

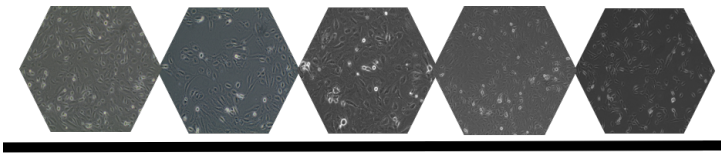
assessed. This was accompanied by the relative quantification of mTOR, Rictor, Raptor and HIF-2α mRNA expression.

We observed a significant decrease of miRNA-101intracellular levels, in both tumoral cell lines, 786-O and FG-2, when compared with HKC-8 (786-O *vs* HKC-8: P=0.030 and FG-2 *vs* HKC-8: P=0.003). Moreover, this miRNA presented higher extracellular levels in FG-2 (P<0.001) and presented a tendency to be higher in 786-O (P=0.052). Additionally, the expression of miRNA-101 intracellular levels presented a tendency to be lower in the resistant cell line (786-OR) when compared with 786-O (P=0.064). This was accompanied by an increase of miRNA-101 expression in 786-OR medium (786-OR *vs* 786-O: P=0.004). Thus, along the resistance acquisition to everolimus, the cells started to excrete more miRNA-101.

Regarding Rictor levels, we observed a significant difference between 786-O and HKC-8 cell lines with a fold-increase of 3.36 (P<0.001). However, we observed a decrease of this mRNA in FG-2 cell line (FG-2 *vs* HKC-8: P=0.031). Additionally, the Rictor expression in 786-OR decreased when compared with 786-O (P=0.003). Regarding Raptor levels, we found an increase of this mRNA expression in both tumoral cell lines, 786-O and FG-2, when compared with HKC-8 (786-O *vs* HKC-8: P<0.001 and FG-2 *vs* HKC8: P=0.08). We also observed a significant decrease of this mRNA in 786-OR when compared with 786-O (P<0.001). In relation to HIF-2α expression levels, we found a significant increase of this mRNA in both tumoral cell lines, 786-O and FG-2, when compared with HKC-8 (786-O *vs* HKC-8: P<0.001 and FG-2 *vs* HKC-8: P=0.016). This was accompanied by an increase of HIF-2α expression in 786-OR when compared with 786-O (P=0.003).

According to our results both mTOR complexes (mTORC1 and mTORC2) seem to be affected by everolimus treatment, but not completely inhibited, which may be associated with the development of resistance. Another major contributor to the acquisition of the resistant phenotype seems to be HIF-2α elevation.

In conclusion, miRNA-101 is a potential predictive biomarker of resistance to mTOR inhibitors since it is excreted by everolimus resistant cells. In the future, the circulating levels of miRNA-101 analysis in blood samples may allow an improvement in monitorization of everolimus resistance acquisition in ccRCC patients submitted to mTOR inhibitors.



Introduction

1.Introduction

1.1 Cancer: general concepts

Cancer is the second leading cause of death worldwide [1]. According to the World Health Organization (WHO), this disease was responsible for 8.8 million deaths in 2015 and the number of new cases is expected to rise in about 70% over the next two decades [2]. In fact, in the last year's cancer incidence has been increasing and it is expected that this number will exceed the 20 million in 2025. This scenario may be due to the population ageing and to the exposure to several risk factors that promote carcinogenesis such as: tobacco consumption, alcohol, unhealthy diet and physical inactivity [3, 4].

Cancer is considered a heterogeneous disease that develops through interactions between environmental and genetic factors, involving dysregulation of multiple pathways responsible for the fundamental cell process, such as death, proliferation, differentiation and cell migration [3]. Thus, the cancer-forming process, called carcinogenesis, is a multifactorial and multiphasic process, which is associated with genetic and epigenetic modifications that promote the development of a malignant neoplasia [5]. Carcinogenesis can be divided into three main phases: initiation, promotion and progression [6]. In the first phase, it occurs different damages in cells in deoxyribonucleic acid (DNA). These damages may have origin in different types of carcinogenic factors such as tumor-promoting chemicals, biological and/ or viruses [7]. Subsequently, promotion is a reversible process that involves a selective clonal expansion of initiated cells leading to the growth and malignant conversion. During this phase, additional genomic and epigenomic lesions occur. Finally, the genetic changes that underlie oncogenesis alter several fundamental properties of cells, conferring the full cancer phenotype. There are two main classes of genes implicated in the development of cancer: oncogenes and tumor-suppressor genes [5]. Proto-oncogenes encode growth-promoting signal molecules and their receptors, anti-apoptotic proteins and some transcription factors. Conversion, or activation, of a proto-oncogene into an oncogene generally involves a gain-of-function mutation. Tumor-suppressor genes normally inhibits cell growth, so mutations that inactivate them allow inappropriate cell division [8, 9].

Cancer cells also acquire other properties that give them an advantage over the normal ones, such as higher proliferative capacity, angiogenesis and invasive potential [10, 11]. In fact, Hanahan and Weinberg proposed a set of cellular characteristics that allow the differentiation of the tumor cells known as the Hallmarks of cancer. Initially, six fundamental cellular properties were defined: sustaining proliferative signaling, evading to growth suppressors, resisting cell death, enabling replicative immortality, angiogenesis induction and invasion and metastasis activation [11]. More recently these authors added new Hallmarks such as: the capacity to avoid immune destruction, inflammation promotion, metabolism dysregulation and genetic instability which promote tumor development (Figure 1) [10].

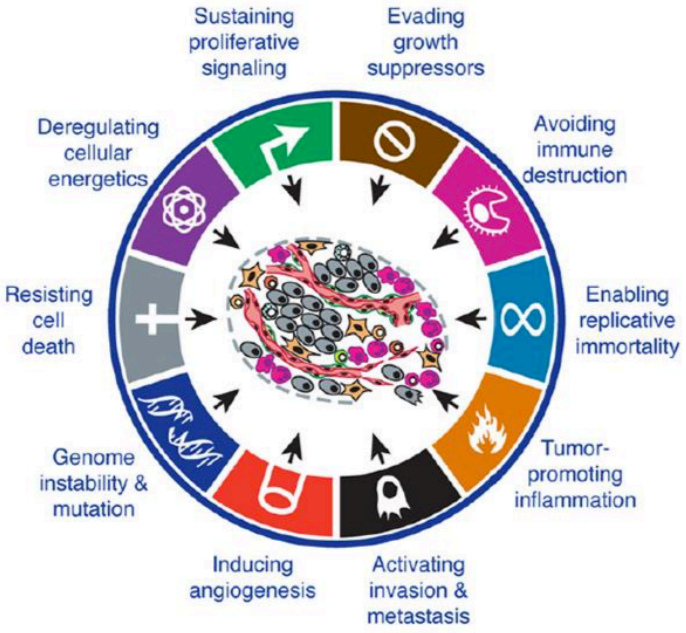


Figure 1 – The Hallmarks of Cancer (adapted from Hanahan, D. and Weinberg, R. 2011) [10].

Despite the advances in the early diagnosis and in the development of new treatments, it still difficult to control cancer development. Thus, the constant evolution in cancer research is an important need, to understand the tumor progression and the nature of tumor cell interactions in their microenvironment, which ultimately lead to the development of new anti-neoplasic therapeutic agents and approaches.

1.2 Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common renal malignancy, representing approximately 2% of all cancers, and it is the mostly lethal urological cancer [12]. Kidney cancer accounts for approximately 84.000 new cases and 35.000 deaths in Europe every year [13]. Actually, there is a 2:1 male predominance, with a peak incidence between 60 and 70 years [13]. According to RORENO (*Registo Oncológico Regional do Norte*), in 2010 kidney cancer became part of the 10 most incidence cancers in the North of Portugal, which shows the incidence increase in the last years [14]. RORENO also estimates that there will be 451 new cases of kidney cancer by 2020 opposing to the 286 cases stated in 2008 [15]. Moreover, RCC incidence and mortality rates presents a geographic variation. The highest incidence rates are observed in the Northern America, Western Europe and Australia, whereas the lowest are observed in India, China and Africa. In relation to mortality rates they are higher in the Central and Eastern countries of Europe and they are lower in Middle and Western Africa (Figure 2) [16].

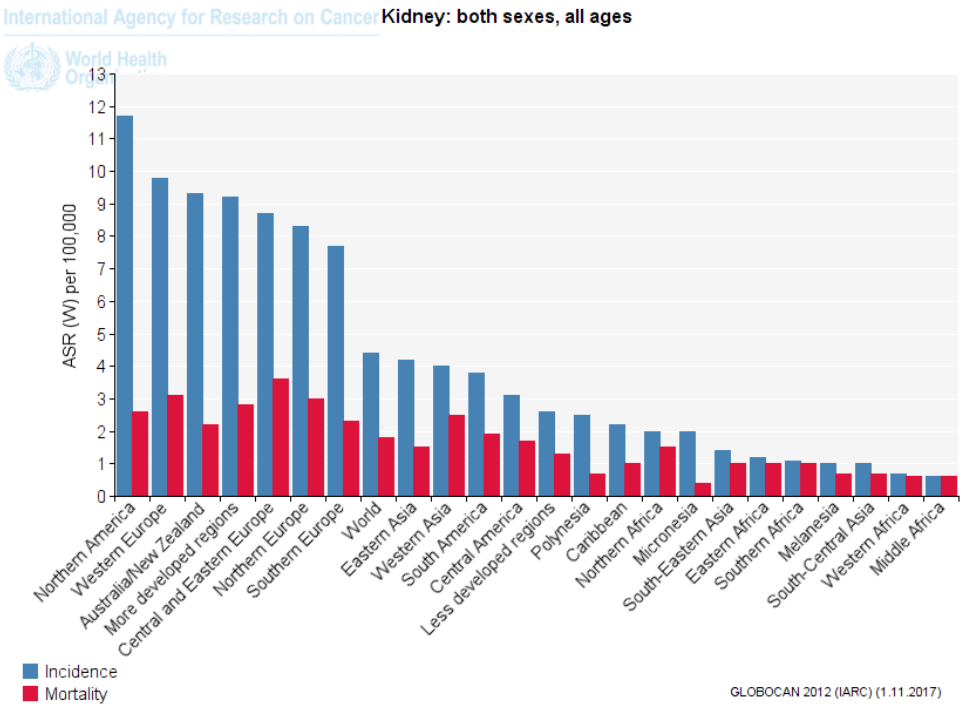


Figure 2 – Distribution of incidence and mortality rates of kidney cancer (Globocan 2012, IARC).

Nowadays, the majority of RCC diagnoses can result from incidental findings due to the anatomical location of the kidney. These *"incidentalomas"* are a consequence of the use of radiological techniques, such as ultrasound and computer tomography performed for other clinical purposes [13]. Additionally, the anatomic location of the kidney is also responsible for the fact that renal masses remain asymptomatic and non-palpable until the late stages of the disease. Patients with worse prognosis are diagnosed after presenting flank pain, gross haematuria and a palpable abdominal mass [17].

RCC is a heterogeneous cancer which is divided into various subtypes, each derived from a different part of nephron with different genetic and molecular alterations, histological features, clinical phenotypes and prognosis [18]. The major subtypes are the clear cell RCC (ccRCC), papillary RCC (pRCC) and chromophobe RCC (chRCC) [19, 20]. The most common subtype of RCC is ccRCC, accounting for approximately 80% of all cases, and it is histologically characterized by high cell lipid content and richly vascularized tumor stroma [21]. ccRCC is the most aggressive subtype and it is associated with a high risk of metastasis formation [22]. The staging of each patient was made according to the AJCC TNM classification system. The TNM staging system is based on primary tumors (T), the involvement of lymph nodes (N) and whether the tumour has metastasized (M) [13]. The figure 3 represents the stages of RCC and management options of each stage.

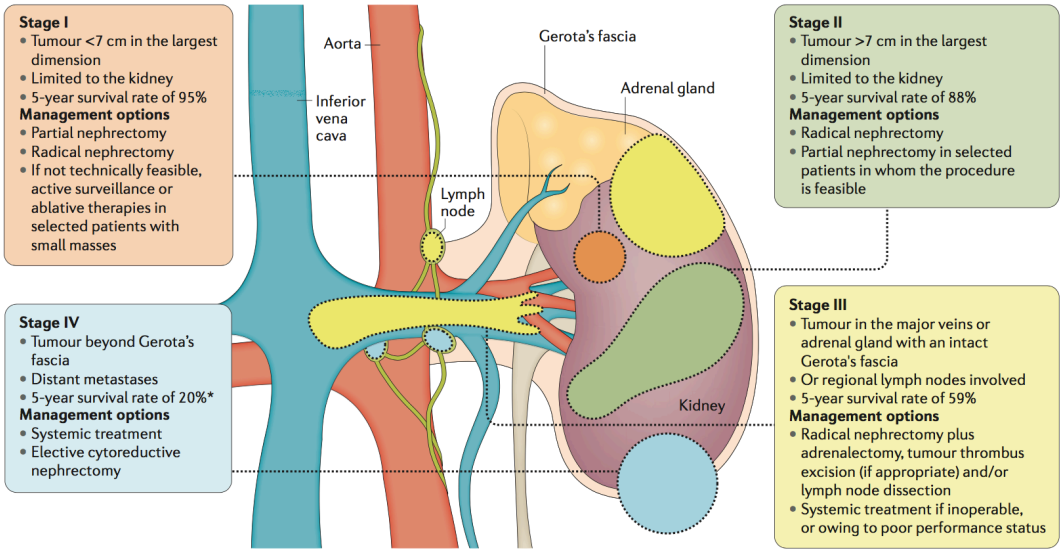


Figure 3 – The stages of RCC and management options (adapted from Hsieh, J. J., *et al*. 2017) [13].

The classification system most widely accepted for histologic grading of RCC is the Fuhrman nuclear grade. The Fuhrman grading system is based on assessment of the uniformity of nuclear size, nuclear shape and nucleolar prominence. Fuhrman nuclear grades 1-2 are associated with tumors in initial stages of development and better prognosis and 3-4 with worst prognosis due to a greater tumor malignancy and aggressiveness [23].

According to the European Association of Urology, the standard treatment of RCC is surgical excision by either partial or radical nephrectomy for patients with surgically resectable tumors [24]. However, approximately 30% of patients initially present with already metastasized disease, and 20-40% of the patients with localized RCC will present disease progression [25]. In cases of patients with inoperable or metastatic RCC the treatment resides in the use of target therapies related with key signaling pathways deregulated in RCC (mTOR and angiogenesis inhibitors) and/or immune check-point inhibitors [26, 27].

One of the signaling pathways involved in the pathophysiology of ccRCC is the *von Hippel-Lindau* (VHL) pathway. In normoxic conditions, the protein encoded by the *VHL* gene serves as recognition site for the regulatory subunits of hypoxia-inducible factors (HIF), targeting them with ubiquitin to proteasome degradation (Figure 4A) [28]. In consequence of the alterations of *VHL gene*, due to the loss of the short arm of chromosome 3, or its inactivation or mutation, the degradation of HIF stops and leads to its accumulation in the cytoplasm and further migration to the nucleus, where it binds to hypoxia-related genes, leading to a cell hypoxic response in normoxic conditions also known as "pseudo-hypoxia" [28]. HIF is composed of a α subunit (HIF-1α, HIF-2α and HIF-3α subunits) and a β subunit (HIF-1β/ARNT). Whereas HIF-1β is constitutively present, the HIF-α members are highly unstable [29]. These alterations lead to the transcriptional activation of genes involved in pathways responsible for angiogenesis and cell growth, such as the transcription of the platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Figure 4B) [29].

It has also been described that HIF expression is dependent of mTOR (Mechanistic target of rapamycin), another molecule involved in ccRCC development [30]. The mTOR protein is a highly-conserved serine/threonine kinase that belongs to the phosphoinositide 3-Kinase (PI3K) – related kinase family. The mTOR signaling pathway integrates both, intracellular and extracellular signals, and serves as a central

regulator of cell metabolism, growth, proliferation and survival [31]. mTOR is a downstream effector of the PI3K/AKT pathway and it is also activated by genetic alterations that reduce the function of the tumor suppressor protein phosphatase and tensin homolog (PTEN) or increase the function of the catalytic subunit of PI3K leading to abnormal activation of AKT leading to activation of mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2) (Figure 4C) [32]. Deregulation of these pathways contributes to the aggressiveness of RCC, allowing the development of new therapies directed to these pathways in the last years.

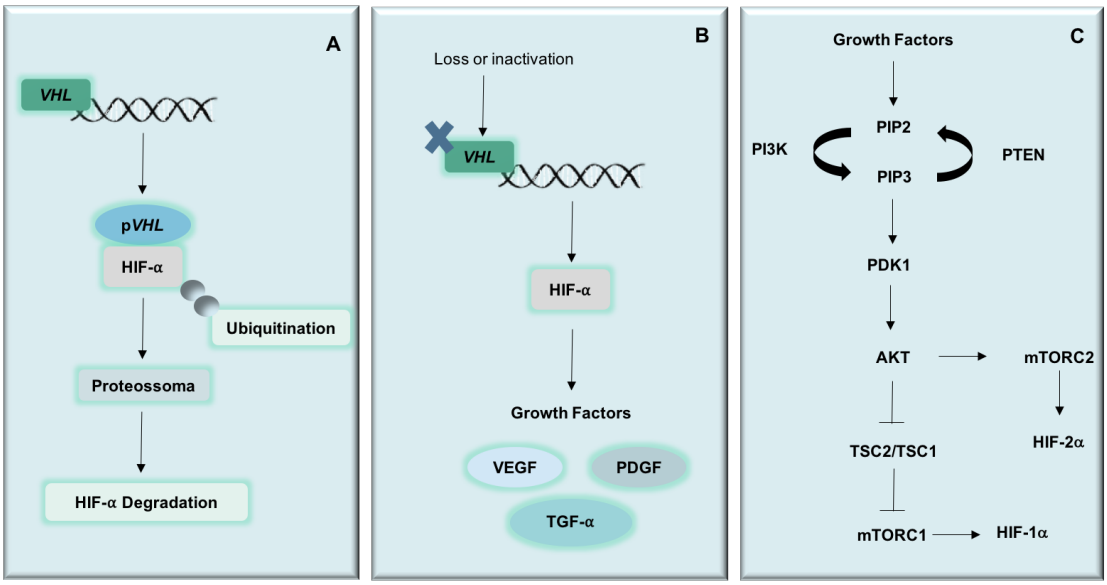


Figure 4 - Signaling pathways involved in renal cell carcinoma pathophysiology. (A) pVHL pathway under normoxic conditions. (B) pVHL pathway under hypoxic conditions. (C) PI3K pathway activation leading to HIF-α expression (adapted from Dias, F., *et al*. 2013) [58].

RCC is traditionally characterized as chemo- and radio-resistant because of the high vascular nature of these tumors and constitutive hypoxic state, respectively, which leads to a poor prognosis reinforcing the need of new treatment approaches [33].

Actually, there are two major types of target agents used in advanced RCC treatment according to the key pathways deregulated: angiogenesis inhibitors that targeted the VEGF ligand (Bevacizumab) or VEGF receptors (VEGFR) (such as the tyrosine kinase inhibitors (TKIs): Axitinib, Sunitinib, Pazopanib and Sorafenib) and inhibitors of the mTOR signaling pathway (Everolimus and Temsirolimus) (Figure 5) [19, 34].

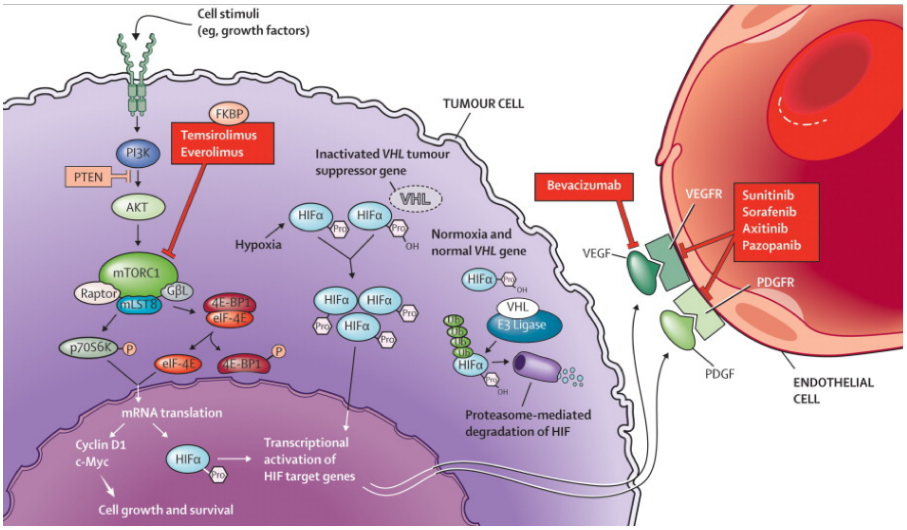


Figure 5 – Biological pathways targeted for therapy in RCC (adapted from Rini, B. I. & Atkins, M. B. 2009) [44].

In terms of therapy response, Ravaud and co-workers divided the patients in three groups: there is a subset of patients, approximately 25%, that are resistant to therapy when they were initially assessed for response after 2–3 months of therapy; a larger group of patients experience tumour regression initially, followed by a short period of disease stability and then disease progression after 6–12 months of treatment and, ultimately, there is a subset of patients that experience tumour regression during the first few months of therapy followed by a longer period of disease stability with no new lesions appearing [35].

Additionally, cytokine therapies (IL-2 and IFN-α) constitute an alternative to the standard metastatic RCC treatment. Interferon alpha (IFN-α) and/or Interleukin 2 (IL-2), activate diverse immune effector cells have improved disease control rates and clinical outcomes. However, the significant toxicities of cytokines and the fact that they present less clinical efficacy when compared with TKIs limits the use of these therapies [36, 37]. More recently, a new treatment approach has been developed, which modulates the immune system against tumour cells. This treatment targets the programmed death 1 (PD-1) receptor which include Nivolumab and Pembrolizumab and its ligand (PD-L1), which include Avelumab and Atezolizumab leading to inhibition of the PD-1 checkpoint pathway [38]. PD1 negatively regulates T cell function and its ligand PDL1 is highly expressed by cancer cells, thus blockade of the PD1-PDL1 axis promotes T cell activation and immune killing of the cancer. However,

the response to Nivolumab only occurs in 25% of patients and others treated patients' did not experience significant tumour reduction [39].

In fact, it is important to note that, despite the prognosis improvement of metastatic RCC patients and the variety of target therapies available, the treatment response is varied and the majority of patients will eventually present disease progression [12]. Metastatic disease remains incurable mainly due to the toxicity profiles and the development of resistances [40]. Additionally, several questions remain unanswered in terms of the alternative sequences treatment and the benefits of using multiple agents or even overcoming treatment resistance.

1.2.1 Resistant mechanisms to target therapies

The target agents approved for RCC treatment have increased the progression free survival and the overall survival [41]. However, these treatments have specific toxicity profiles, which can lead to dose reduction and even discontinuation of the treatment [42]. On the other hand, cytokine therapies only present benefit in a small subset of patients (generally those with intrinsically favourable disease biology) and are associated with substantial toxicity, particularly in the case of IL-2 use [12, 37].

In general, the resistance to target therapies develops, when a tumor becomes independent from the activity of drug targeted pathway [43]. It may happen due to several mechanisms (Figure 6). According to Malouf and co-workers the advances in RCC therapeutic schemes have led to resistance to first-line treatments, such as resistance to TKIs [30]. The resistance to VEGFR inhibitors is often due to mutation in a gene encoding a key receptor tyrosine kinase. However, these mutations would have to take place in the tumour endothelium, which is the main target of VEGFR inhibitors and it is almost impossible that identical mutation coexists on each individual tumour metastasis [44].

Another potential resistance mechanism is the upregulation of alternative proteins and/or pathways that re-establish angiogenesis and growth capacity in an independent VEGF manner [44]. For example, alternative angiogenesis can be triggered by the upregulation of angiopoietin 2, c-MET (hepatocyte growth factor receptor) or IL-8 (Interleukin-8) signaling, whereas the proliferation can be promoted through upregulation of the PI3K/AKT/mTOR pathway [30].

Other resistance mechanism involves the occurrence of an increased drug efflux which can results in a decreased intracellular TKIs concentrations [40, 45]. TKIs are captured and stored in intracellular compartments instead of reaching cancer cells promoting low concentrations in plasma and serum. Inflammation promotion is another mechanism of resistance. Bone marrow derived cells modulate expression of a wide variety of cytokines, growth factors and enzymes. They are recruited as a result of hypoxia occurrence, which in turn is caused by vascular regression mainly due to anti-angiogenic therapy. This type of cells has the ability to create new blood vessels promoting tumor adaptation and resistance to targeted therapies [43].

Regarding the mTOR inhibitors, they form a complex with the FK binding protein (FKBP) and inhibit mTOR from complex 1 (mTORC1). However, the other mTOR complex, the mTOR complex 2 (mTORC2) may not be inhibited by these targeted therapies [44]. This fact suggests that inhibition of mTORC1 may leads to a compensatory activation of PI3K/AKT pathway via upregulation of mTORC2, which will activate AKT and HIF-2α, limiting the therapeutic effect of mTOR inhibitors [40].

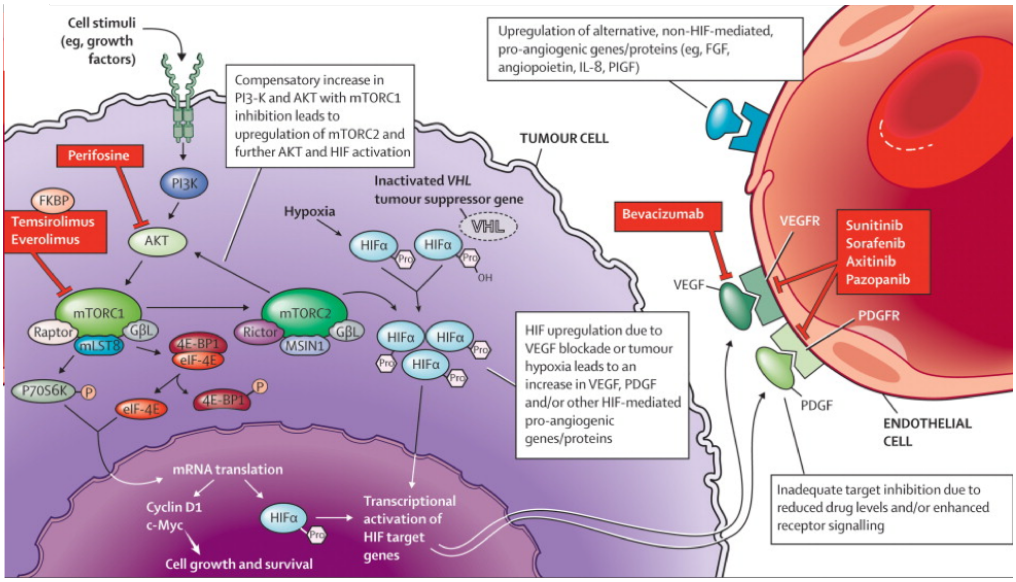


Figure 6 – Mechanisms of resistance to target therapies of RCC (adapted from Rini, B. I. & Atkins, M. B. 2009) [44].

The mTOR inhibitors act inside the tumor cell and they are the first line of treatment in patients with poor prognosis [37, 46]. Therefore, it is important to study the molecular pathology of RCC and PI3K/AKT/mTOR pathway involvement to understand these mechanisms of resistance.

1.3 PI3K/AKT/mTOR pathway

The PI3K (*phosphatidylinositol 3-kinase*) pathway is a signal transduction cascade that is responsible for many physiological functions, including cell cycle, cell survival, protein synthesis, metabolism, motility and angiogenesis [32, 47]. The PI3K family is divided into four different classes: Class I, Class II, Class III and Class IV. Class I are heterodimeric molecules composed by a regulatory subunit: PIK3R1, PIK3R2 and PIK3R3 and a catalytic subunit: PIK3CA, PIK3CB and PIK3CD. The class IV is composed by ATM (ataxia telangiectasia mutated), DNA-PKcs (DNA-dependent protein kinase) and mTOR (mammalian target of rapamycin) [48]. PI3K converts its substrate phosphatidylinositol 4,5-biphosphate–PI (4,5) P2 into PI (3,4,5) P3 (or PIP3). However, PTEN antagonizes PI3K activity and negatively regulates Protein Kinase B signaling. Protein Kinase B, also known as AKT is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes [31].

AKT can appear in one of three isoforms (AKT1, AKT2, AKT3) [31]. Phosphoinositide-dependent kinase 1 (PDK1) is recruited to the membrane and phosphorylates AKT at Ser308 and mTORC2 phosphorylates AKT at Ser473 promoting full activation of AKT [49]. AKT inhibits through phosphorylation TSC1/TSC2 (tuberous sclerosis complex). TSC1/2 functions as a GTPase-activating protein (GAP) and negatively regulates mTORC1 signaling by converting Rheb (Ras homolog enriched in brain) into its inactive form. The active form of Rheb directly interacts with mTORC1 to stimulate its activity [50-52].

mTOR is a downstream effector of the PI3K/AKT pathway and the catalytic subunit of the two biochemically distinct complexes, called mTORC1 and mTORC2 (Figure 7) [53]. mTORC1 has five components: mTOR, which is the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec 13 protein 8 (mLST8, also known GβL); proline-rich AKT substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (Deptor). mTORC2 comprises six different proteins, some of them common to mTORC1: mTOR; rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1); mLST8 and Deptor [32, 54]. It is important to note that each complex has its own

protein composition, which reflects their differences in terms of upstream signal integration, substrate regulation and biological process control.

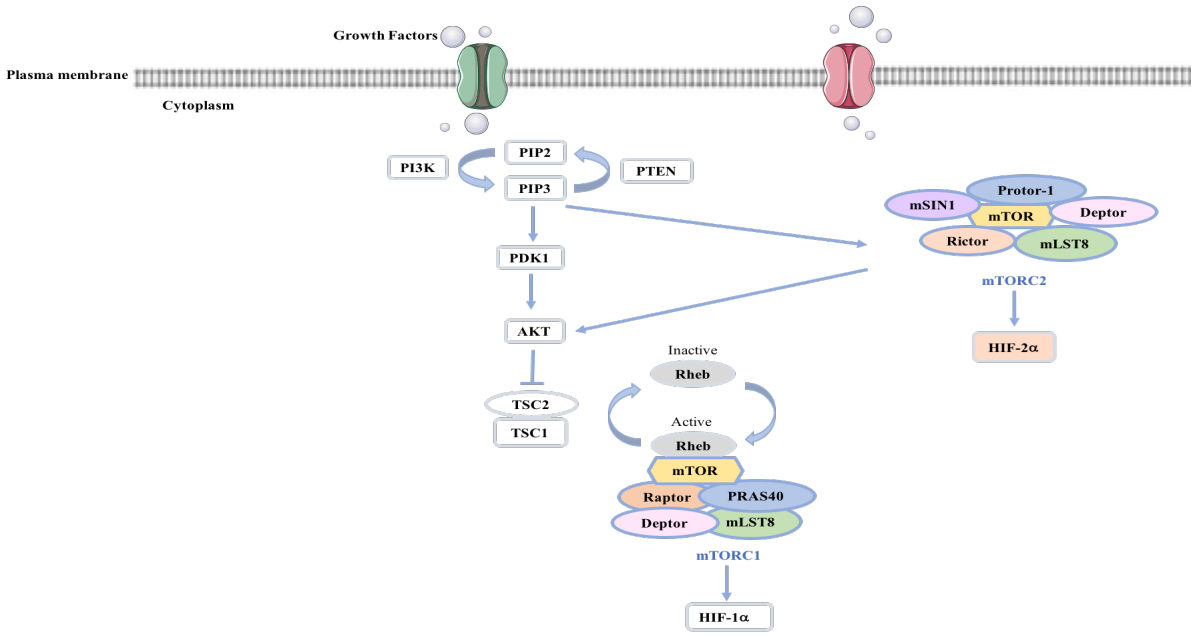


Figure 7 – Schematic representation of the PI3K/AKT/mTOR pathway and its relation with HIF. It is interesting to note that the different HIF subunits are related to different mTOR complexes: HIf-1α is regulated by mTORC1 while HIF-2α is regulated by mTORC2 (adapted from Nogueira I., *et al.* 2018) [69].

mTORC1 is regulated by nutrients, growth factors, cellular energy and stress pathways while mTORC2 is primarily regulated by growth factors [32, 50]. Additionally, mTORC1 and mTORC2 are both positively regulated by interferons (IFNs). The mTOR complexes have distinct functions: mTORC1 positively regulates cell growth and proliferation by promoting many anabolic processes, including biosynthesis of proteins, lipids and organelles and by limiting catabolic processes such as autophagy while mTORC2 plays key roles in various biological processes, including cell survival, metabolism, proliferation and cytoskeleton organization [31]. mTOR signaling is often deregulated in ccRCC, which can lead to an up regulation of HIF and a worse patients' prognosis [31, 55]. Additionally, it is important to identify new targets and consequently, the development of new forms of treatment. Several studies described the involvement of microRNAs (miRNAs) dysregulation in the pathogenesis and progression of ccRCC [56]. These small molecules can be considered as potential diagnostic and prognostic biomarkers, allowing disease monitorization, as well as promising new therapeutic agents [40].

1.4 MicroRNAs

MiRNAs are a class of short non-coding RNA (ncRNA) molecules (approximately 22 nucleotides of length) that regulate gene expression at the post-transcriptional level, by binding to the complementary region of corresponding mRNAs targets, leading to the inhibition or degradation of mRNAs [57, 58].

The primary transcripts of miRNAs are produced in the nucleus. Primary miRNAs (pri-miRNA) are transcribed by RNA polymerase II and they are processing by Drosha and its cofactor DGCR8, creating a pre-miRNA [59, 60] . The pre-miRNAs are carried out to the cytoplasm by the nuclear export protein (XPO5), where it is cleaved by Dicer to generate the mature double-stranded miRNA [61]. The mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) which will guide them to the complementary region of their targets and this process results in the inhibition of mRNA (messenger ribonucleic acid) translation, or promotes its degradation and leads to post-transcriptional gene silencing (Figure 8) [62-64] .

Recent publications suggest that miRNAs have an important role in cancer development, influencing all cancer hallmarks [10]. miRNA expression is dynamic since a miRNA can regulate more than one mRNA while different miRNAs can interact with the same mRNA [65]. Several studies have shown that miRNAs are expressed or inhibited in different types of cancer, suggesting that miRNAs work as onco-miRNAs and downregulating tumor suppressor genes, being overexpressed in cancer cells or tumor suppressor miRNA, downregulating oncogenes, being under-expressed in cancer [57, 66, 67]

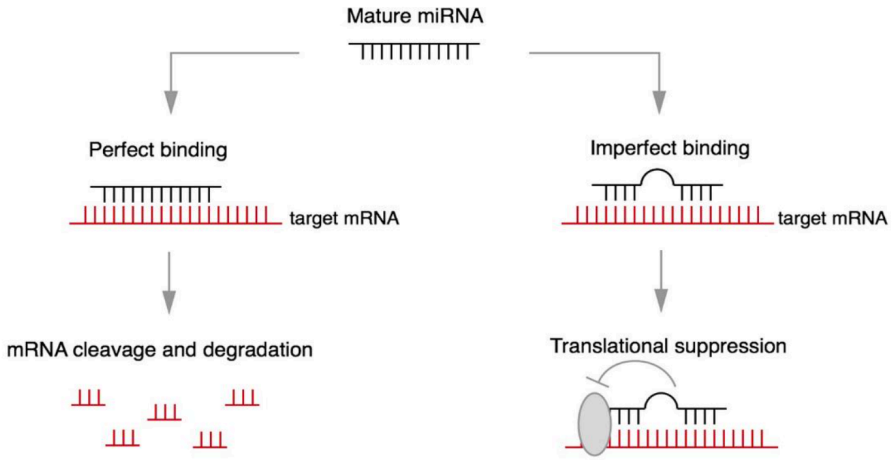


Figure 8 – MicroRNAs regulation mechanisms (adapted from Teixeira, A.L., *et al*. 2014) [57].

The circulating levels of specific miRNAs are promising noninvasive blood-based biomarkers with a high potential for the early RCC diagnosis and monitorization of therapy response since they may be predictive of resistance therapies. For example, according to a previous publication from our group, the increased plasma levels of miRNA-221 has a key role in cellular microenvironment, modulating important cellular processes involved in carcinogenesis and cancer progression. This study reports that patients with higher plasma miRNA-221 levels had a significantly lower survival rate and a high risk of death by RCC [68].

Since miRNAs may be associated with pathogenesis of RCC and may be predictive of resistance therapies it would be important to study the role of miRNAs in acquiring resistance to mTOR inhibitors.

1.4.1 MicroRNAs as regulators of PI3K/AKT/mTOR pathway

The PI3K/AKT/mTOR pathway, is perhaps the most commonly activated signaling pathway in human cancer. Bibliographic review was done in order to understand which miRNAs are associated with this pathway (Attachment 1) [69]. Nowadays, it is known that miRNAs have an important role in regulation of mTOR signaling in most cancer types (Figure 9).

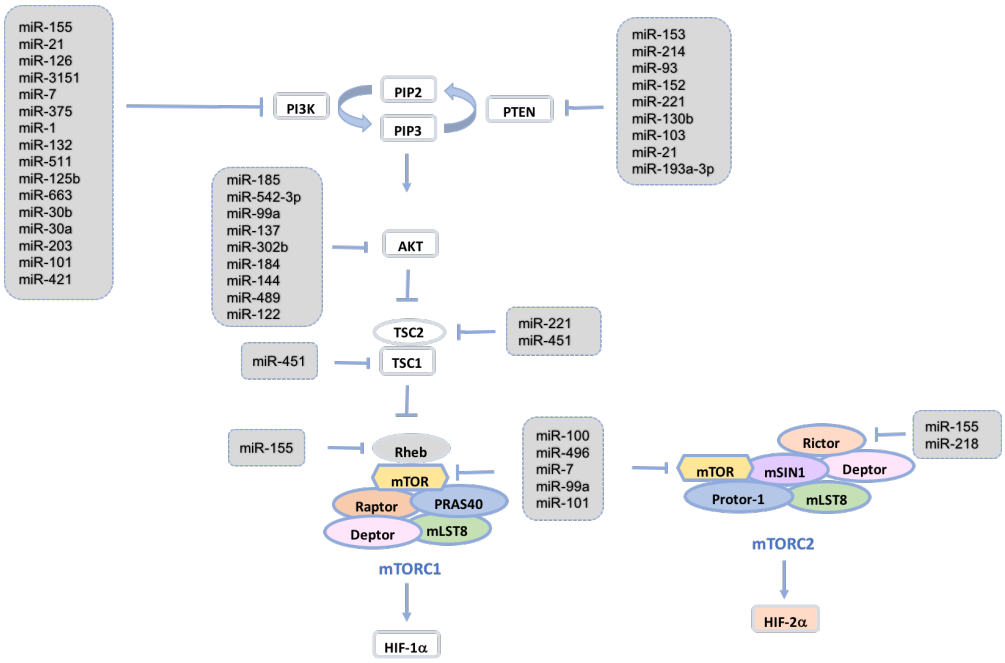
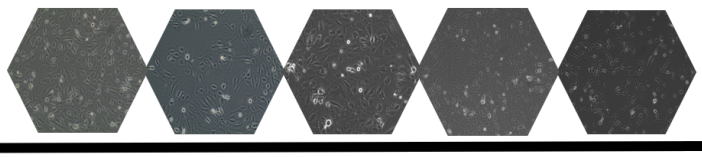


Figure 9 – Schematic representation of the microRNAs that are involved in the regulation of PI3K/AKT/mTOR pathway in cancer (adapted from Nogueira I., *et al.* 2018) [69].

PI3K/AKT/mTOR pathway is induced as a consequence of one the first molecular events associated with RCC, the HIF deregulation, and miRNAs have been clearly demonstrated to have relationships with mTOR signaling pathway in RCC [70].

Zheng and co-workers showed that miRNA-101 level was significantly lower in human RCC tissues and cell lines and inhibits indirectly the mTOR pathway by targeting DNA-PKcs, a member of PI3K, and that regulates mTORC2 activation [71].

Because mTOR is a validated therapeutic target for RCC, and the clinical practice is facing some issues due to de development of resistance to anti-mTOR therapies, circulating miRNAs, may be used to predict the resistance to these therapies since may be detected by a liquid biopsy [67, 70]. Therefore, it would be interesting to study the miRNAs deregulated in RCC that can be used as prognostic biomarkers. Additionally, they can be used as predictive of resistance allowing changings in the therapeutic approach, with a significant impact in patients' overall survival.



Objectives

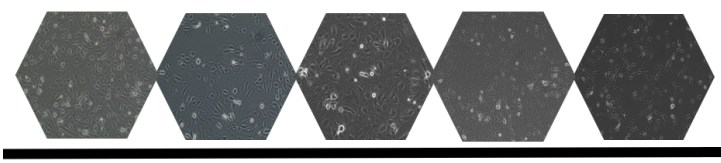
2.Objectives

2.1 Main Objective

MicroRNA-101 and associated levels of mTORC2 and HIF-2α in resistance development to mTOR inhibitors in ccRCC.

2.2 Specific Objectives

- *In vitro* quantification of miRNA-101 levels (intra- and extracellulary) in renal cell carcinoma cell lines (FG-2 and 786-O) *versus* primary renal proximal tubule epithelial cell line (HKC-8);
- *In vitro* quantification of HIF-2α, Rictor, Raptor and mTOR mRNA in renal cell carcinoma cell lines (FG-2 and 786-O) *versus* primary renal proximal tubule epithelial cell line (HKC-8);
- Establishment of an everolimus-resistant renal cell carcinoma cell line (786-OR);
- Evaluate the potential of miRNA-101 as a molecular biomarker of resistance acquisition to everolimus;
- *In vitro* quantification of HIF-2α, Rictor, Raptor and mTOR mRNA in 786-OR.



Material and Methods

3.Material and Methods

3.1 Cell line characterization and cell culture

Three cell lines were used to perform the present study: HKC-8, 786-O and FG-2 (Figure 10). The HKC-8 cell line is a human-derived normal renal proximal epithelial tubular cell line. The 786-O is a renal cell adenocarcinoma cell line derived from a 58 years old man and FG-2 is derived from a 77 years old man and it is described as a metastatic RCC cell line. Both HKC-8 and FG-2 were kindly provided by Professor Klaas Kok from Groningem University, Netherlands and the 786-O cell line was kindly provided by Professor Cármen Jerónimo from the Epigenetics and Cancer Biology Group of CI-IPO-Porto.

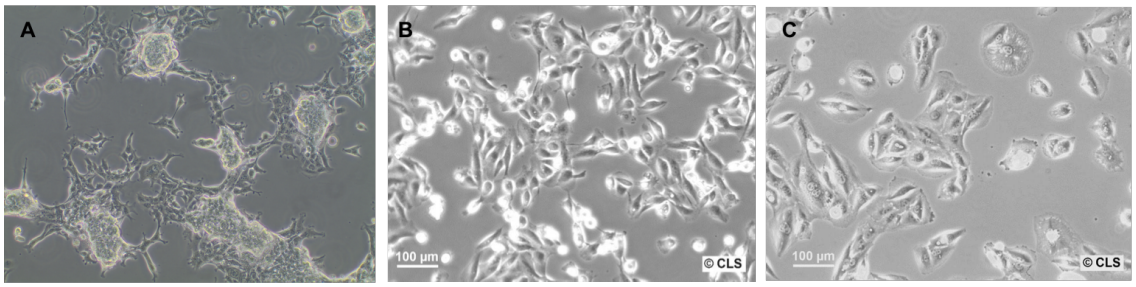


Figure 10 – (A) Microscope image of the HKC-8 cell line (Photograph taken using an Olympus IX51 microscope). (B) Microscope image of the 786-O cell line (Cell Lines Service © CLS). (C) Microscope image of the and FG-2 cell line. (Cell Lines Service © CLS).

Initially a cryopreserved vial of each cell line was thawed. Both 786-O and FG-2 cells were maintained in RPMI 1640 (1X) medium (*Gibco*®), supplemented with 10% of FBS (Fetal Bovine Serum) (*Gibco*®) and 1% of Pen-Strep (penicillium-stretomycin mixture) (*Gibco*®). HKC-8 cells were maintained in DMEM/F12 medium (*Gibco*®), supplemented with ITS (Insulin-transferrine-selenum) (*Sigma-Aldrich*®), Pen-Strep (*Gibco*®), EGF (Epidermal Growth Factor) (*Sigma-Aldrich*®), Hepes buffer (*Gibco*®) and Hydrocortisone (*Sigma-Aldrich*®). The three cell lines were kept in an incubator with the following conditions: 37ºC of temperature, 5% CO2 and humid atmosphere.

3.2 Establishment of an everolimus-resistant renal cell carcinoma cell line

Firstly, a cryopreserved vial of the 786-O cell line was thawed. An everolimus-resistant subline (786-OR) was generated upon exposure of the 786-O cell line to crescent concentrations of everolimus (*Afinator® 10 mg - Novartis*). One everolimus pill was diluted in 10.4 mL of DMSO (Dimethyl sulfoxide, *SIGMA®*) to obtain a 10mM stock solution. In terms of exposure times and everolimus concentrations, we adapted a previously established protocol for target therapy resistance induction in RCC cell lines [72]. Specifically, 15 days exposure to everolimus at 5μM, followed by 15 days exposure to everolimus at 10μM and finally 2 months exposure to everolimus at 20μM.

In order to confirm that the resistance establishment was successful we performed a cell viability assay using Resazurin Sodium Salt (*ACROS Organics™ – fisher scientific)*. Resazurin is a cell permeable redox indicator that can be used to monitor viable cells. Continued growth is associated with a reduced cell environment while inhibition of growth maintains an oxidized environment. The resazurin change from oxidized form (non-fluorescent, blue) to its reduced form resorufin (fluorescent, red) when it is observed a reduction of cell growth. The data regarding oxidized/reduced forms of resazurin can be collected using either fluorescence-based or absorbance-based methodology. Absorbance is monitored at 570nm and 600nm and was the method chosen for this work. The two variables that most affect the response of cells to resazurin are the length of incubation time and number of cells plated. The optimum plating density and incubation time should be determined for each cell line since each cell line presents distinct growth features. We determined both these variables for the 786-O cell line, being the best plating density 40 000 cells/well and the best incubation time of 3 hours.

After the protocol optimizations, we analyzed the cell viability of the 786-O and the established 786-OR cell lines in order to confirm the resistance acquisition phenotype to the everolimus resistance. In a 96-multi well plate, 40 000 cells/well of 786-O and 786-OR were cultured in 8 wells each. After 24 hours, Resazurin was added to each well in a 10% proportion to the final volume. Absorbance's were then measured at 570 nm and 600 nm upon 3h of Resazurin addition using a plate reader *FLUOstar Omega*, *BMG Labtech, Offenburg, Germany*. This experiment was performed at the end

of 15 days exposure to everolimus at 5μM, 15 days exposure to everolimus at 10μM and 15 days exposure to everolimus at 20μM, 1 month exposure to everolimus at 20μM and at the end of the establishment of the everolimus-resistant renal cell carcinoma cell line.

In order to calculate the percent difference in reduction of resazurin between treated (786-OR) and control cells (786-O) we used the following formula:

$$\frac{(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of test agent dilution}}{(\varepsilon_{OX})\lambda_2 A°\lambda_1 - (\varepsilon_{OX})\lambda_1 A°\lambda_2 \text{ of untreated positive growth control}} \times 100$$

Figure 11 – Formula to calculate the percent difference in reduction between treated and control cells in metabolic capacity experiment. ε_{OX}-molar extinction coefficient of Resazurin oxidized form; A-absorbance of test wells; A°- absorbance of positive growth control well; λ_1- 570nm; λ_2-600nm.

3.3 MicroRNA and mRNA extraction and cDNA synthesis

When the desired cell confluence was achieved (80-90%) the medium, in which the cells were being cultured, was collected for miRNA extraction and the cells were trypsinized, using 0.05 % trypsin-EDTA (1×) (*Gibco*®) and counted using EVE™ Automated Cell Counter (*NanoEnTek*®) and Tripan-Blue dye (*Gibco*®). After counting, approximately 2 million cells were centrifuged to form a pellet for either miRNA or mRNA extraction and the remaining cells were kept in culture.

This procedure was repeated five times for each cell line and respective medium. Taking in consideration the 786-OR cell line, this procedure was performed at the end of the 15 days exposure to everolimus at 10μM and at the end of the establishment of the everolimus-resistant cell line.

MicroRNA extraction (from the cells and respective medium) was performed using the GRS microRNA kit (*Grisp*®) and mRNA extraction (from the cells) was performed using the GRS Total Blood & Cultured Cells Kit (*Grisp*®), according to a procedure already optimized in our lab [68, 73].

The miRNA samples were then used as templates for cDNA synthesis using a Taqman®MicroRNA Reverse Transcription kit (*Applied Biosystems*®) and sequence-specific stem-loop primers for miRNA-101, RNU-44, RNU-48 and RNU-6B and the mRNA samples were then used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (*Applied Biosystems*®). After protocol optimization, the thermal conditions were as follows: 16ºC for 30 min, followed by 42ºC for 60 min and 85ºC for 10 min for miRNAs cDNA synthesis and 25ºC for 10 min, followed by 37ºC for 120 min and 85ºC for 5 min for mRNA.

3.4 Real-time PCR relative quantification

The miRNA and mRNA expression was analyzed by quantitative real-time PCR. The reactions were carried out on a StepOne™qPCR Real-Time PCR and StepOnePlus™ qPCR Real-Time PCR machine, containing 1X Master mix (*Applied Biosystems®*), with 1X probes (*TaqMan® microRNA Expression Assays*, miRNA-101*: TM-002143, *TaqMan® microRNA Control Assays*, RNU-6B: TM-001093, RNU-48:

TM-001006 and RNU44: TM-001094 or *TaqMan® mRNA Expression Assays*, HIF-2α: HS01026149 and human GUSB (Beta Glucuronidase, (*Applied Biosystems®*) and a cDNA sample. RNU-6B, RNU-44 and RNU-48 were used and quantified to determine which one had the most constant expression levels in the microRNA tested samples to normalize results and function as endogenous control. According to a previous study from our group, GUSB was described as the best endogenous control for mRNA normalization in the same RCC cell lines [73]. Therefore, this endogenous control was used in the present study.

According to Sarbassov *et al* and Masri *et al*, mTORC1 and mTORC2 expression can be indirectly quantified. Thus, mTORC1 and mTORC2 can be represented by the mRNA expression of specific molecules of each complex, such as Raptor for mTORC1, Rictor for mTORC2 and mTOR is common to both complexes. The mTOR complexes were analyzed according to their relative levels of Raptor, Rictor and mTOR. Quantitation of the relative levels of cDNAs encoding mTOR, Rictor, Raptor and β-Actin (ACTB) was performed using Fast SYBR green master mix (*Applied Biosystems®*). β-Actin was used as an endogenous control to mRNA tested samples. The primer sequences used are presented in table 1 [74]. PCR conditions were as follows: 95ºC for 20s, 95ºC for 3s, 63,5ºC (mTOR)/64,5ºC (Rictor)/66ºC (Raptor)/63ºC (ACTB) for 30s, 95ºC for 15s, 60ºC for 1min and 95ºC for 15s.

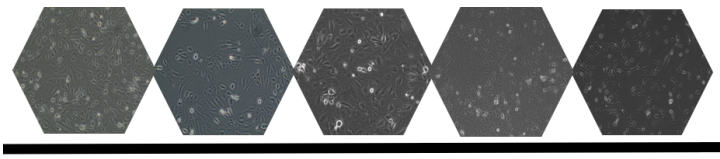
Table 1 – Primer sequence of mTOR, Rictor, Raptor and ACTB used in relative quantification with Fast SYBR green master mix.

Gene	Primer sequence
mTOR	5'-GAC TGC TTT GAG GTT GCT ATG AC-3' 5'-CCT TTG GTA TTT GTG TCC ATC AGC-3'
Rictor	5'-AAC ACC AAG CAG GTT CAT GAA AGC-3' 5'-CAG ATG GAA GAC CTC CTG CAT CA-3';
Raptor	5'-TGA CGG CCA CAG ACG ATG GTG CC-3' 5'-CGT AGG GAT GTC CTG CAC CTT CA-3'
β-Actin	5'- CTA AGT CAT AGT CCG CCT AGA AGC A-3' 5'- TGC CAC CCA CGA CAA TGA A-3'

Data analysis was performed using *StepOne™ Sofware v2.2* (*Applied Biosystems®*) and the baseline and threshold were set for each plate to create threshold cycle (CT) values for all the miRNAs and mRNAs in each sample. All quantifications were performed in duplicate and each plate had a negative control.

3.5 Statistical Analysis

Statistical analysis was performed using *IBM® SPSS® Statistics software* for *Windows* (version 22.0). Livak method ($2^{-\Delta\Delta Ct}$) and t´ student test was used to evaluate the differences in the expression levels of the normalized miRNAs and mRNAs.



Results

4.Results

4.1 Endogenous control selection for microRNAs relative quantification

The graphs represented in figure 12 show the Ct (the cycle number at which the fluorescence generated within a reaction crosses the background fluorescence) mean value of RNU44, RNU48 and RNU-6B endogenous controls, both in cells (intracellular) and in culture medium (extracellular). The results show that RNU44 is the endogenous control that presents more constant and stable mean Ct values and smaller standard deviations. As so, RNU44 was the endogenous control chosen to normalize the miRNA expression levels in the present study.

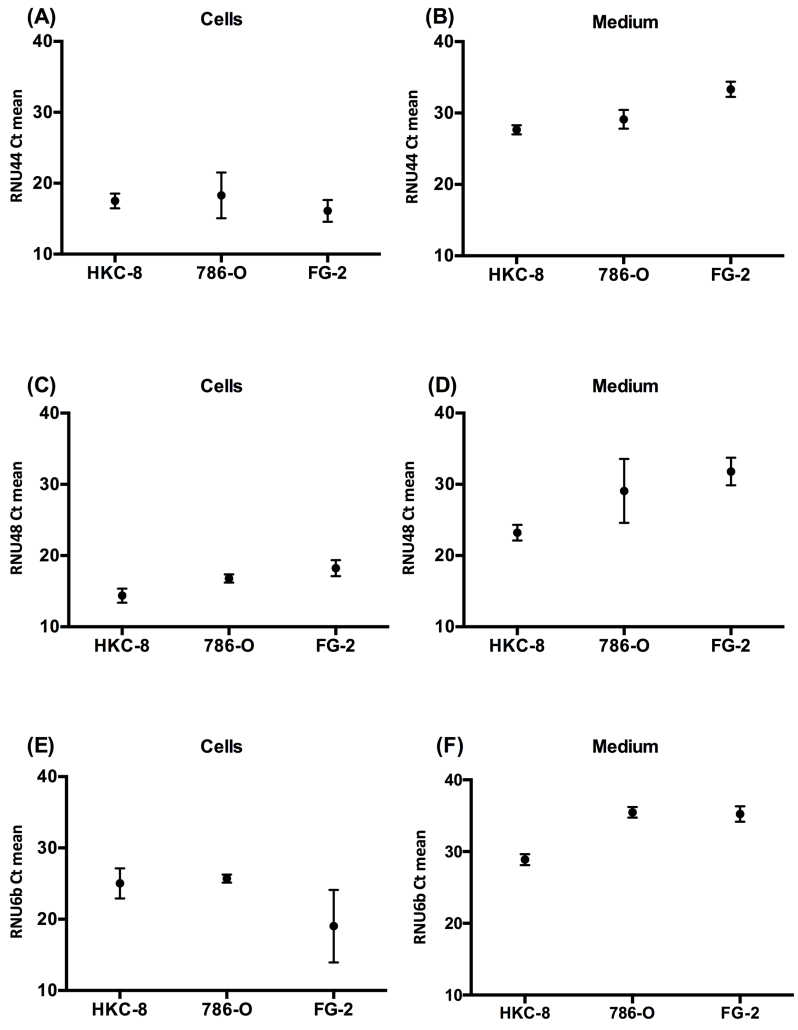


Figure 12 – Mean Ct values of three human miRNAs endogenous controls analyzed in the three renal human cell lines used in the study (A, C and E) and in the corresponding culture medium (B, D and F).

4.2 MiRNA-101, HIF-2α, Rictor, Raptor and mTOR mRNA basal levels in HKC-8, 786-O and FG-2 cell lines

Figure 13 shows the graphs representing the intra (A) and extracellular (B) levels of miRNA-101. According to the results, there is a significant difference in the intracellular levels of miRNA-101 when comparing HKC-8, 786-O and FG-2 cell lines. In fact, it has been observed a fold-decrease of 0.36 in the 786-O cell line compared to HKC-8 (fold change= 0.36, *P*=0.030). The same tendency is observed when comparing HKC-8 and FG-2 cell lines, with a fold change of 0.26 in the FG-2 cell line (fold change= 0.26, *P*=0.003) (Figure 13 A).

Regarding the extracellular levels, the expression of the miRNA-101 is significantly higher in FG-2 medium (fold change=46.21, *P*<0.001) and present a tendency to be higher in 786-O medium (fold change=3.16, *P*=0.052) compared to the HKC-8 cell medium (Figure 13 B).

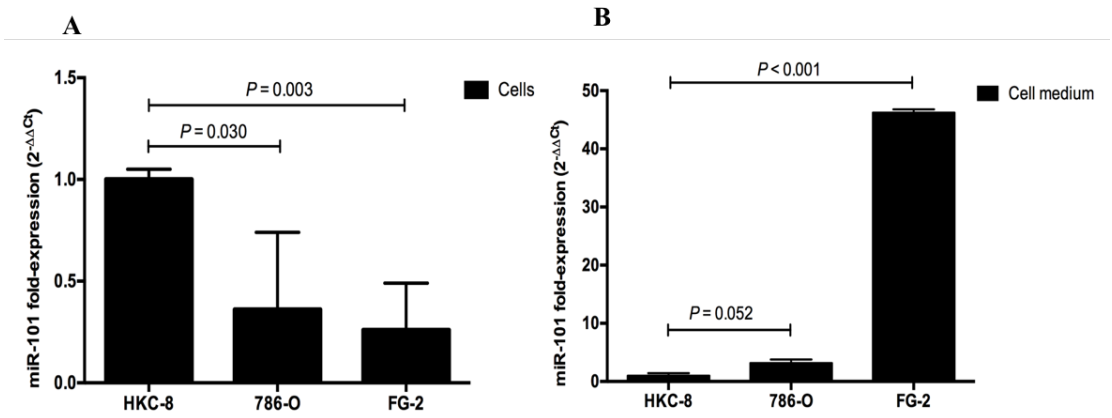


Figure 13 – MiRNA-101intracellular (A) and extracellular (B) expression levels in 786-O and FG-2 cell lines compared to HKC-8 cell line.

Regarding the HIF-2α mRNA expression levels, we observed significantly higher levels of this transcript in 786-O (fold change= 10.1, *P*<0.001) and in FG-2 (fold change= 1.72, *P*=0.016) when compared with HKC-8 cell line (Figure 14).

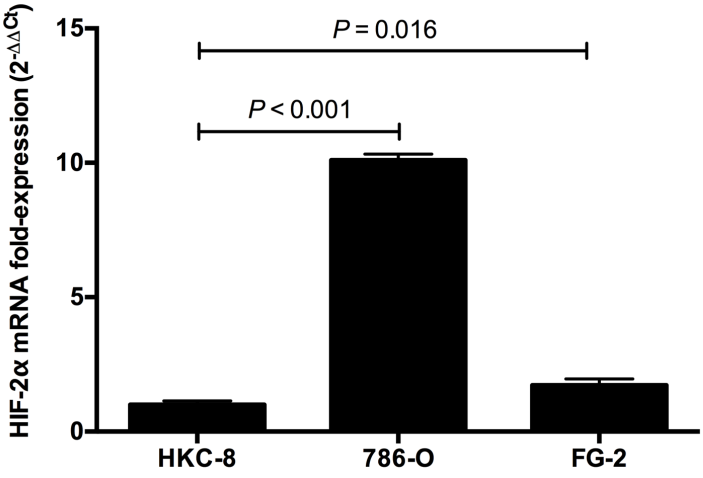


Figure 14 – HIF-2α mRNA levels change in 786-O and FG-2 cell lines compared to HKC-8 cell line.

Figure 15 shows the graphs representing the mTOR, Raptor and Rictor mRNA levels. According to the results, there is also a significant difference in mTOR mRNA levels between HKC-8 and 786-O cell lines, with a fold-increase of 5.46 in 786-O cell line (fold change= 5.46, *P*<0.001). However, there is no statistical significant difference in mTOR mRNA levels in HKC-8 *versus* FG-2 cell lines (fold change= 0.37, *P*=0.057) (Figure 15A).

Regarding the Raptor mRNA levels, we observed significantly higher levels of this transcript in 786-O cell line (fold change= 9.99, *P*<0.001) when compared with HKC-8 cell line. However, there is no statistical significant difference in Raptor mRNA levels between HKC-8 and FG-2 cell lines (fold change= 2.60, *P*=0.08) (Figure 15B).

According to the results, there is also an increase of Rictor mRNA in 786-O cell line with a fold-increase of 3.36 when compared with HKC-8 (fold change= 3.36, *P*<0.001). Regarding the FG-2 cell line there is a decrease of Rictor mRNA expression when compared with HKC-8 (fold change= 0.29, *P*=0.031) (Figure 15C).

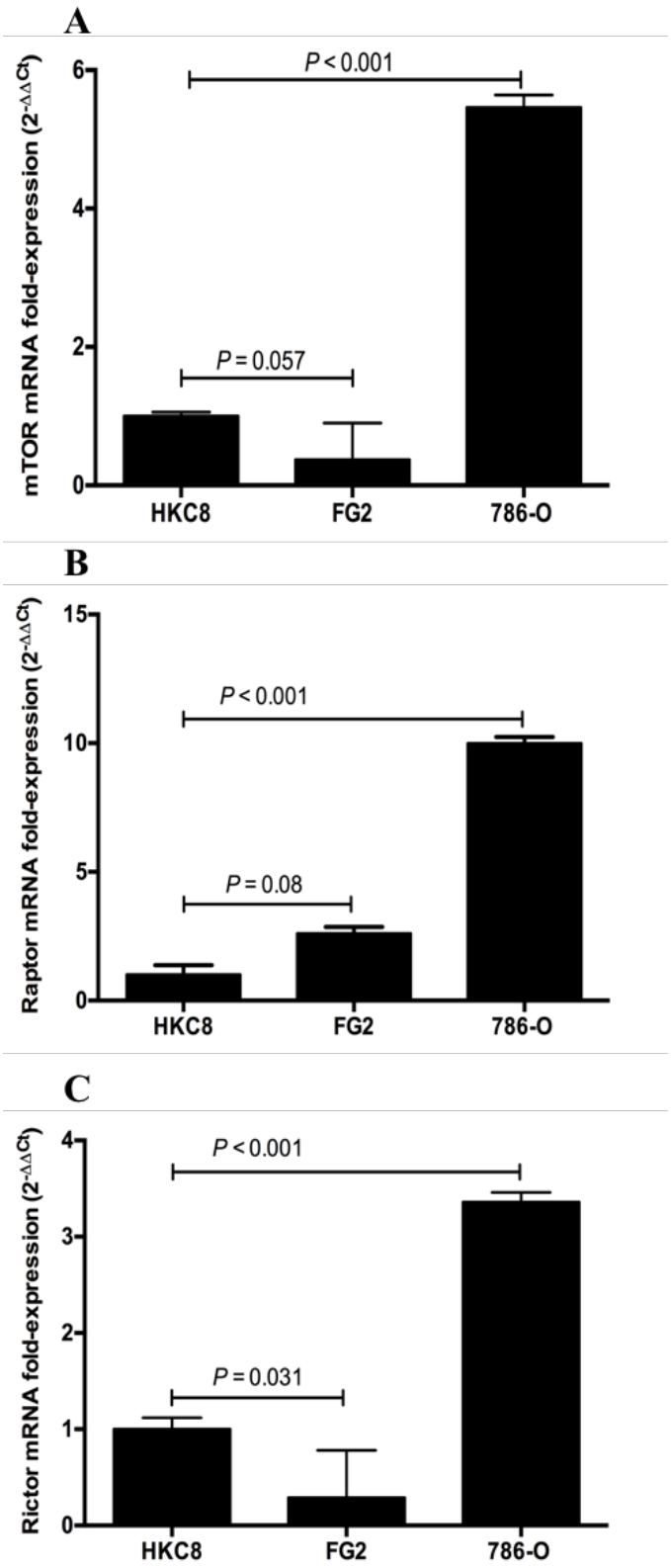


Figure 15 – mTOR (A), Raptor (B) and Rictor (C) mRNA levels variation in 786-O and FG-2 cell lines compared to HKC-8 cell line.

4.3 Characterization of an everolimus-resistant RCC cell line

In figure 16 is represented the graph describing the difference in resazurin reduction capacity between everolimus treated cells (786-OR) and control cells (786-O) at different time points.

According to the results, at the end of 15 days of exposure to 5μM of everolimus, there were no differences in cell viability between 786-O and 786-OR. However, at the end of 15 days of exposure to 10μM of everolimus there was a decrease of 30% of metabolic capacity in the 786-OR comparatively to 786-O cells. However, after this time period at 10μM of everolimus exposure, the 786-OR cells started to recover their metabolic capacity and reached the 100% of metabolic capacity after 15 days of exposure to everolimus at a concentration of 20μM. After that time point, the 786-OR cells were able to maintain their metabolic capacity during 2 months in same exposure conditions, which indicates that the cells successfully developed resistance to everolimus.

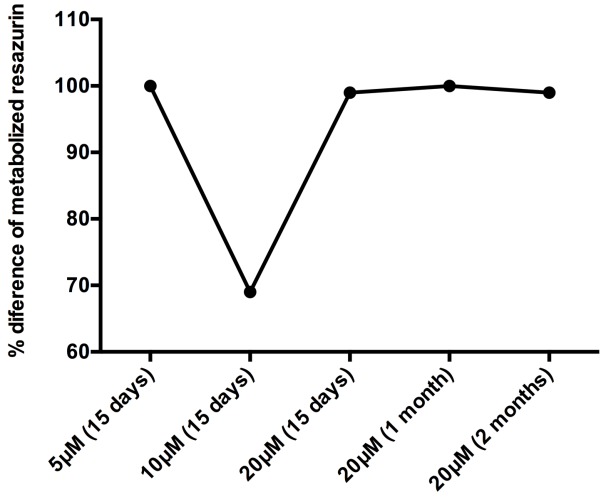


Figure 16 – Metabolic capacity of 786-OR cells at different time points submitted to different everolimus concentrations.

In figure 17, the microscope images of 786-O (Figure 17A) and 786-OR (Figure 17 B-F) shows morphological changes at different stages of resistant phenotype establishment. There is a significant difference in 786-OR morphology after 15 days of exposure to everolimus at 5μM (Figure 17B) and the same difference remains after 15 days of exposure to everolimus at 10μM (Figure 17C) and after 15 days of exposure to everolimus at 20μM (Figure 17D) when compared with control cells. After 1 month of exposure to everolimus at 20μM the cells begin to acquire their normal morphology (Figure 17E). Finally, after 2 months of exposure to everolimus at 20 μM, 786-OR seems to have the same morphology as 786-O (Figure 17F).

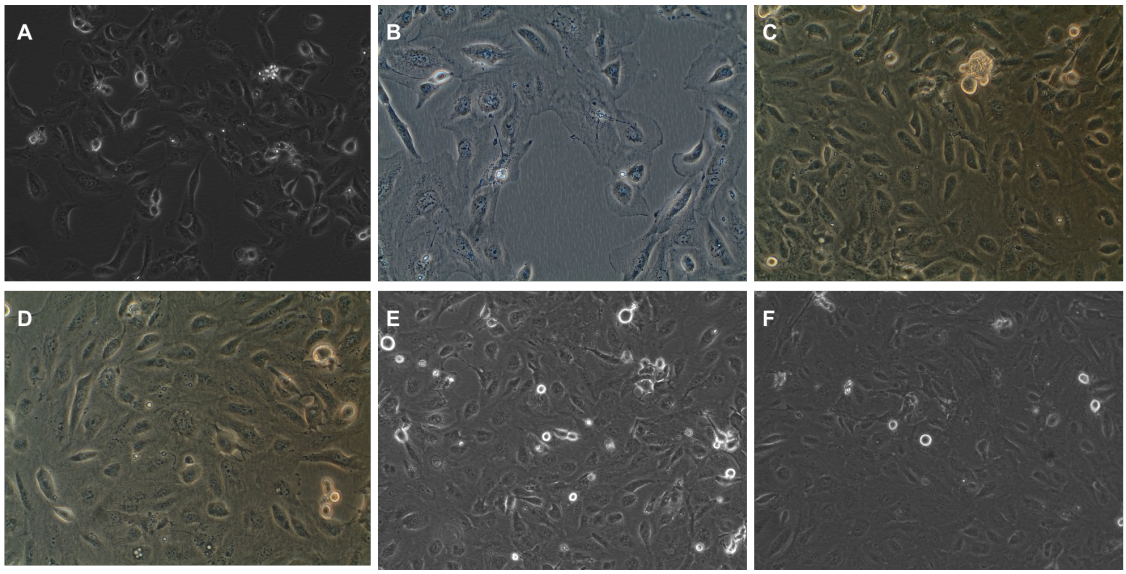


Figure 17 – Microscope images of 786-O (A) cells and treated cells (786-OR) (20x) at different everolimus concentration and time points. (B) 786-OR after 15 days of exposure to everolimus at 5μM. (C) 786-OR after 15 days of exposure to everolimus at 10μM. (D) 786-OR after 15 days of exposure to everolimus at 20μM. (E) 786-OR after 1 month of exposure to everolimus at 20μM. (F) 786-OR after 2 months of exposure to everolimus at 20μM.

4.4 786-OR cell line: MiRNA-101, HIF-2α, Rictor, Raptor and mTOR mRNA levels

Figure 18 shows the intracellular (A) and extracellular (B) levels of miRNA-101 in 786 cell line during the acquisition of everolimus resistance. According to the results, there is no statistical significant difference in 786-OR after 15 days of exposure to everolimus at 10μM (fold change= 0.42, *P*=0.115) and present a tendency to be lower in 786-OR after 2 months of exposure to everolimus at 20μM (fold change= 0.33, *P*=0.064) (Figure 18A).

Regarding the extracellular levels, when compared with the miRNA-101 levels in 786-O medium with 786-OR medium after 15 days of exposure to everolimus at 10μM there is no statistical significant difference (fold change= 0.62, *P*=0.557). However, the expression of miRNA-101 is significantly higher in 786-OR medium after 2 months of exposure to 20 μM everolimus when compared to the 786-O medium, with a 11.63 fold-increase in expression levels (fold change=11.63, *P*=0.004) (Figure 18B).

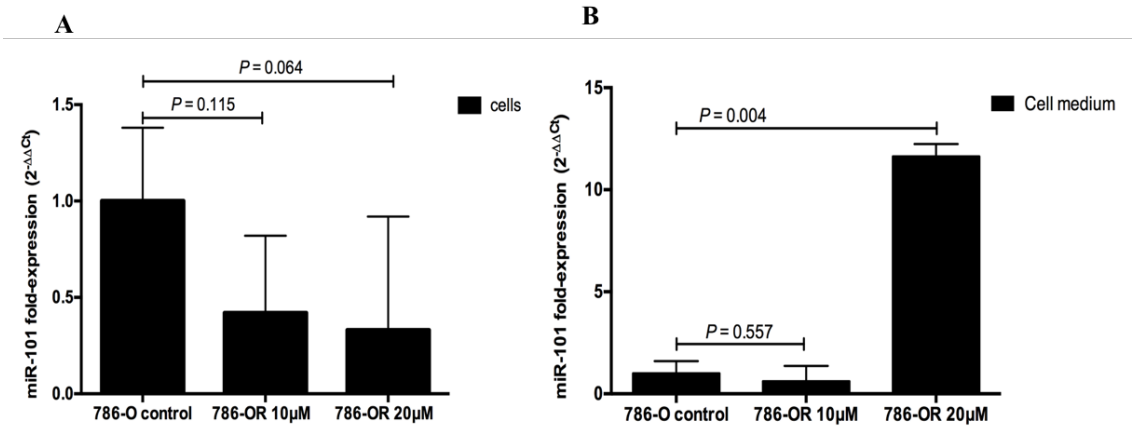


Figure 18 – Variation of the intracellular (A) and extracellular (B) expression levels of miRNA-101 in 786-O, 786-O after 15 days of exposure to everolimus at 10μM and 786-O after 2 months of exposure to everolimus at 20μM.

Regarding the HIF-2α mRNA expression levels, we observed that after 15 days of exposure to everolimus at 10μM there was a decrease of HIF-2α mRNA expression in 786-OR (fold change= 0.29, *P*=0.005) when compared with 786-O. However, we observed an increase of HIF-2α expression in 786-OR after 2 months of exposure to everolimus at 20μM (fold change= 2.83, *P*=0.003) when compared with 786-O (Figure 19).

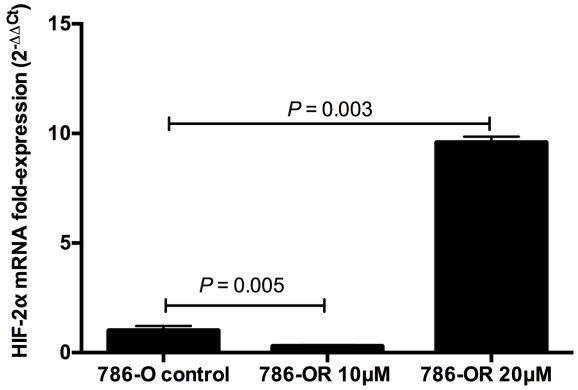


Figure 19 – Variation of the relative expression levels of HIF-2α mRNA in 786-O, 786-O after 15 days of exposure to everolimus at 10μM and 786-O after 2 months of exposure to everolimus at 20μM.

Figure 20 represents the mTOR mRNA relative expression levels at different time points of 786-OR establishment. According to the results, there is no statistical significant difference between 786-O and 786-OR after 15 days of exposure to everolimus at 10μM (fold change= 1.31, *P*=0.083) and after 2 months of exposure to everolimus at 20μM (fold change= 1.02, *P*=0.450).

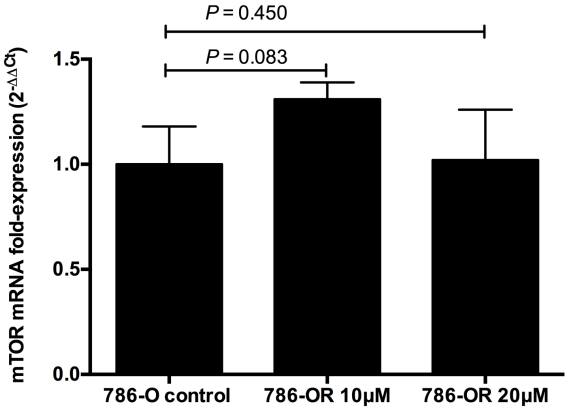


Figure 20 – Variation of the relative expression levels of mTOR mRNA in 786-O, 786-O after 15 days of exposure to everolimus at 10μM and 786-O after 2 months of exposure to everolimus at 20μM.

Regarding the Raptor mRNA relative expression, it is significantly lower in 786-OR after 15 days of exposure to everolimus at 10μM (fold change= 0.08, *P*=0.001) and after 2 months of exposure to everolimus at 20μM (fold change= 0.22, *P*<0.001) when compared with 786-O cell line (Figure 21).

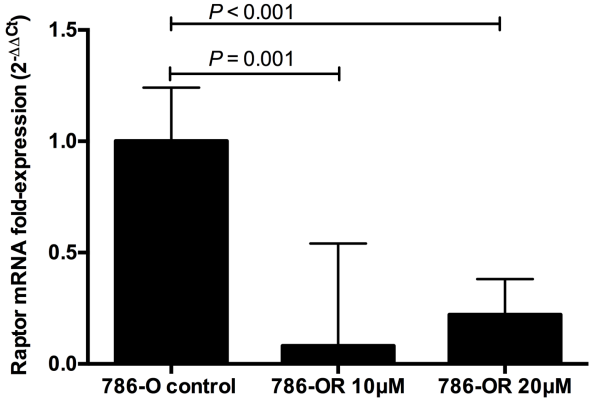


Figure 21 – Variation of the relative expression levels of Raptor mRNA in 786-O, 786-O after 15 days of exposure to everolimus at 10μM and 786-O after 2 months of exposure to everolimus at 20μM.

In figure 22, it is represented the Rictor mRNA relative expression levels. According to the results, there is a decrease in 786-OR after 15 days of exposure to everolimus at 10μM (fold change= 0.04, *P*<0.001) and after 2 months of exposure to everolimus at 20μM (fold change= 0.08, *P*=0.003) when compared with 786-O cell line.

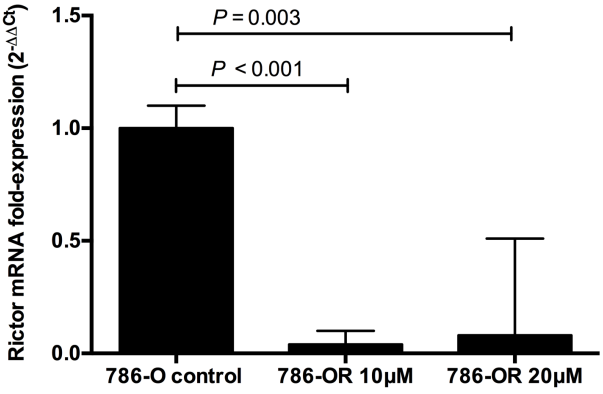
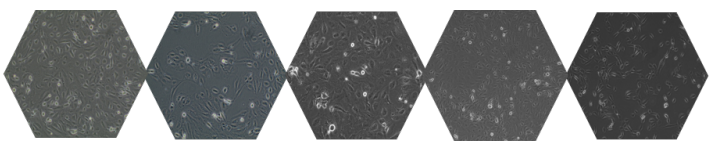


Figure 22 – Variation of the relative expression levels of Rictor mRNA in 786-O, 786-O after 15 days of exposure to everolimus at 10μM and 786-O after 2 months of exposure to everolimus at 20μM.



Discussion

5.Discussion

ccRCC is a neoplasia that presents an aggressive cell phenotype and a high potential to metastasize due to its intense vascularity and to the overexpression of angiogenic factors [13]. One of the major signaling pathways deregulated in ccRCC is the PI3K/AKT/mTOR pathway [75]. This pathway is responsible for several physiological functions, including cell cycle, cell survival, protein synthesis, growth, metabolism, motility and angiogenesis [76, 77].

In the recent years, the discovery of the pathways involved in ccRCC pathogenesis allowed the development of targeted therapies directed to these pathways [41, 78]. One example, it is the everolimus, an mTOR inhibitor used in the treatment of ccRCC patients that present a worse prognosis [26]. This agent forms a complex with the FK binding protein and inhibits mTORC1 [75]. However, despite the improvement in ccRCC outcomes achieved by this targeted therapy, the control of this disease is time-limited, mainly in consequence of the acquisition of resistance to this therapy [40, 79]. Taking this into account, the need of new, effective and predictive biomarkers increase. These biomarkers would be important to predict which patients will develop resistance to these targeted therapies and consequently disease progression, allowing in the future changings in the therapeutic approaches, with a significant impact in patients' clinical outcomes. Since miRNAs are key elements in the regulation of gene expression and because they present a different expression patterns in normal and tumoral tissues, and during the disease progression, they can be used as potential biomarkers in this field [70, 80].

Several studies describe the miRNA-101 as a tumor suppressor miRNA, and as down-regulated in many solid cancers, including hepatic, pancreatic, lung and prostate cancer [69]. In ccRCC, two studies reported its downregulation in cancer cell lines when compared with a normal epithelial kidney cell line [71, 81]. These results are consistent with the results found in the present study, since miRNA-101 is significantly decreased in 786-O and FG-2 cell lines when compared with HKC-8. Additionally, the circulating levels of specific miRNAs are promising noninvasive blood-based biomarkers with a high potential for the early RCC diagnosis and for the monitorization of therapy response. However, there are still no studies reporting miRNA-101 extracellular levels in ccRCC. In fact, we observed for the first time an increased

expression of miRNA-101 in the cell medium of both ccRCC cell lines, which suggests the secretion of this miRNA to the surrounding extracellular environment. Taking this data into account, we hypothesize that, in addition to a decrease in miRNA-101 production by ccRCC cancer cells, the cells may also increase the excretion of this miRNA to the cellular microenvironment. The miRNA-101 excretion, and consequent uptake by the tumor neighbor cells, will result in this miRNA influencing its proliferation suppression functions on these cells, which ultimately will result in a proliferative advantage to the tumor cells.

Since miRNA-101 targets DNA-PKcs, which positively regulates mTORC2 and AKT activation, when miRNA-101 is downregulated there is a downstream activation of PI3K/AKT/mTOR signaling pathway, including mTORC2, which lead to cell survival and proliferation [71]. An indirect method for either mTORC1 and mTORC2 expression levels analysis was proposed by Sarbassov *et al* and Masri *et al* [82, 83]. In this method, mTORC1 and mTORC2 expression can be associated with the mRNA expression of specific molecules of each complex, such as Raptor for mTORC1 and Rictor for mTORC2. Thus, since the mTOR is common to both complexes, we can assume that, when we have higher levels of Raptor, we have a higher expression of mTORC1 and when we have higher levels of Rictor, we have a higher expression of mTORC2.

According to our study, we observed an increase of Rictor expression in 786-O when compared with HKC-8, meaning an increased mTORC2 expression. This is supported by the constitutive hypoxic state described for this cell line, due to the constitutive expression of HIF-2α, since mTORC-2 activates the HIF-2α transcription and consequently the protein expression [84, 85]. However, the expression of Rictor is decreased in FG-2 cell line when compared with HKC-8 and 786-O cell lines. As so, what we propose is that in FG-2 cell line there is a lower expression of mTORC2 probably due to the tendency of mTORC1 upregulation. Regarding Raptor expression, we observed an upregulation of its mRNA in 786-O when compared with HKC-8. As so, we can infer that mTORC1' expression is increased in this tumor cell line. These deregulations were accompanied by the increase of mTOR levels in 786-O cell line when compared with HKC-8 and FG-2 cell lines. These facts validate that the upregulation of either mTORC1 and mTORC2 is a key contributor to the malignancy and more aggressive phenotype of ccRCC.

In order to clarify the role of both mTORC2 and miRNA-101 in the development of resistance to mTOR inhibitors an everolimus-resistant cell line (786-OR) was established. We verified a decreased expression of miRNA-101 intracellular levels in 786-OR when compared with 786-O, accompanied by an increase of its extracellular levels. As so, what we propose is that besides a decrease in miRNA-101 production by everolimus-resistant cell line, there is also an increase of this miRNA excretion. These results are very interesting and highlight the potential use of miRNA-101 as noninvasive blood-based biomarker specifically for the monitorization of mTOR-inhibitors therapy response in ccRCC patients.

In our *in vitro* study, everolimus had an effect in mTORC1 expression since the Raptor mRNA expression decreased in 786-OR cell line along with the acquisition of everolimus resistance when compared with control cells (786-O). When trying to explain the mechanisms of resistance to everolimus, several researchers propose that the resistance to mTOR inhibitors can be explained by a compensatory activation of PI3K/AKT pathway via the upregulation of mTORC2 [86]. However, in our study, we observed that Rictor is significantly decreased in 786-OR cell line. Moreover, regarding the mTOR expression, our results show that mTOR levels remain approximately the same along the acquisition of resistance to everolimus.

The decrease in Rictor expression, and consequently of mTORC2, suggests that the resistance to mTOR inhibitors may not be exclusively dependent of the upregulation of this molecule but can be supported by the overexpression of other pathways, such as HIF-2α pathway. Upon activation, HIF-2α translocate to the nucleus and regulates the expression of a wide range of genes implicated in tumorigenesis [28]. In fact, we observed that both tumoral cell lines the 786-O and FG-2 presented an increase of HIF-2α expression levels when compared with normal cell line HKC-8. These results suggest that HIF-2α has tumorigenic activity in ccRCC. Furthermore, according to our study, HIF-2α is significantly increased in 786-OR cell line when compared with 786-O cell line. As so, what we propose is that the resistance to mTOR inhibitors can be supported by the overexpression of HIF-2α, which can lead to activation of several pathways related with cell survival and proliferation. Chengxing and colleagues, showed that HIF-2α directly and indirectly promotes proliferation through the mitogen-activated protein kinase (MAPK) pathway [87]. Additionally, the PI3K pathway and the MAPK pathways share common upstream activators and these pathways are

significantly interconnected by feedback loops. One pathway provides compensatory signaling when the other is inhibited [88]. Arkaitz Carracedo and colleagues showed that in conditions of mTORC1 inhibition, Ras is activated and signals to MAPK pathways [89, 90]. As so, the upregulation of MAPK pathways may be related with acquired resistance to mTOR inhibitors.

The results of the present study demonstrate that everolimus inhibits mTORC1 and affect the mTORC2 expression. On the other hand, the development of everolimus resistance results in HIF-2α pathway upregulation in ccRCC resistant cell line (Figure 23). Moreover, the results suggest that miRNA-101 is a potential predictive biomarker of resistance to mTOR inhibitors since this miRNA is excreted by the resistant cells' to everolimus (Figure 23). Additionally, the excretion of this miRNA was observed at the end of 3 months while the resistance to mTOR inhibitors in patients, which is detected by disease progression, only was observed at the end of 6-15 months [44]. As so, the circulating levels of miRNA-101 may allow an improvement in the monitorization of everolimus resistance acquisition and in the future new therapeutic approach. Since alterations in this miRNA expression can be detected early, this miRNA can be applied to the clinical practice without a painful procedure to the patient.

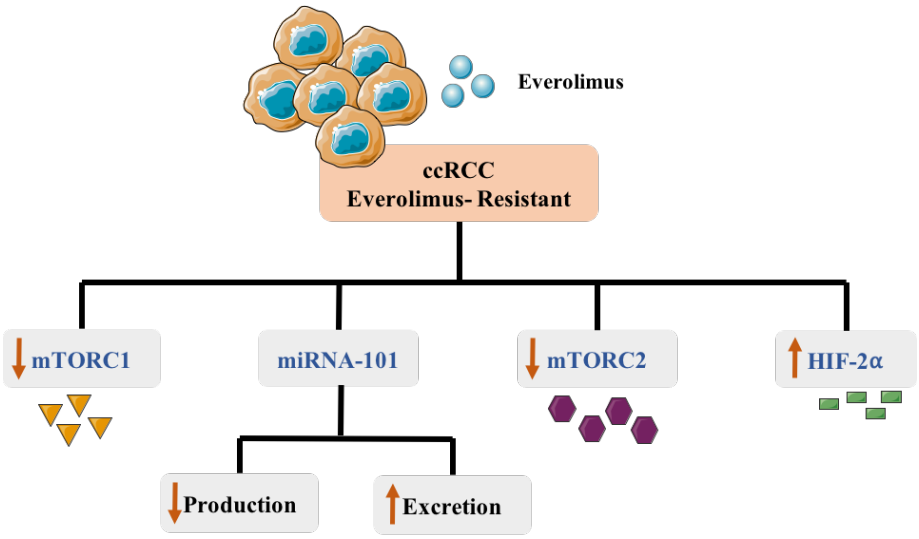
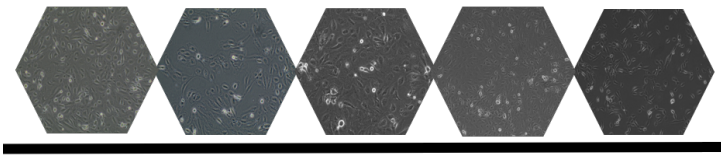


Figure 23 – Proposed model of PI3K/AKT/mTOR and HIF-2α pathways regulation in the development of resistance to mTOR inhibitors in RCC and the role of miRNA-101 during the acquisition of resistance, according to the results obtained in the present study.



Conclusion and Future Perspectives

6.Conclusion and Future Perspectives

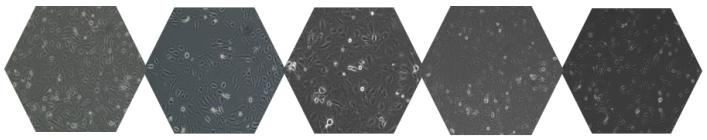
The use of targeted therapies directs to key molecules involved in RCC development has been revolutionizing cancer treatment, however these therapies have limitations mainly due to the acquisition of resistance to them.

Everolimus, an mTOR inhibitor, inhibits the activation of mTORC1 which can lead to a compensatory activation of PI3K/AKT pathway, which potentially can drive resistance via upregulation of mTORC2. According to our *in vitro* study, the resistance to mTOR inhibitors seems not to be related with overexpression of mTORC2, since this complex is downregulated in the resistant cell line established in this study. In fact, the downregulation of mTORC2 suggests that the resistance to mTOR inhibitors may not be exclusively dependent of the upregulation of this complex but can be supported by the overexpression of other pathways, such as HIF-2α pathway, observed in this study. As so, it would be interesting in the future to study another pathway's related to the upregulation of HIF-2α, such as the MAPKs pathway. In addition, it would be useful to quantify the protein levels of the corresponding mRNAs quantified in the present study and other proteins that comprises the complexes, mTORC1 and mTORC2, in order to replicate and validate the results obtained.

MiRNAs may be useful biomarkers to predict targeted therapies resistance since they regulate gene expression of different molecules involved in crucial cellular processes and they are easy to quantify using biological samples. MiRNA-101, which indirectly and positively regulates the mTORC2 activation, seems to be an important regulator of resistance to mTOR inhibitors. In fact, we have found that miRNA-101 expression is deregulated in ccRCC.

This deregulation seems to be established not only by a decrease in the production of miRNA-101 but also by an increase of its excretion in ccRCC cell lines. These results, were also observed in everolimus-resistant cell line. The deregulation of this miRNA and the increase of its excretion in resistant cell line defines it as possible circulating biomarker and offers the possibility to use the miRNA-101 as potential predictive biomarker of resistance to mTOR inhibitors.

According to our results, in the future, it is important to replicate the study *in vivo*, in order to validate the potential of miRNA-101 as circulating biomarker in the clinical practice. In the future studies, it would also be useful to understand what are the differences in the miRNA-101 expression levels of ccRCC patients before initiating therapy with mTOR inhibitors and monitor their expression along the treatment. This would help to clarify the role of this miRNA as possible biomarker, allowing the monitoring of response to therapy predicting the resistance. Additionally, it would be interesting to search for more deregulated miRNAs in ccRCC, related with these pathways in order to establish a miRNA profile.



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7.References

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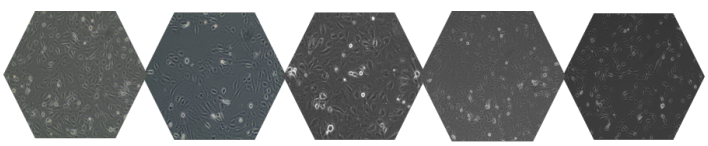

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Review

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Pharmacogenomics

miRNAs as potential regulators of mTOR pathway in renal cell carcinoma

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Renal cell carcinoma (RCC) is the most commonly occurring solid cancer of the adult kidney with the majority of RCC cases being detected accidentally. The most aggressive subtype is clear cell RCC (ccRCC). miRNAs, a family of small noncoding RNAs regulating gene expression have been identified as key biological modulators. The von Hippel–Lindau pathway is one of the signaling pathways involved in the pathophysiology of ccRCC. Another oncogenic mechanism involves the activation of PI3K/AKT/mTOR signaling and serves as a central regulator of cell metabolism, proliferation and survival. Several studies have described the involvement of miRNA dysregulation in the pathogenesis and progression of ccRCC. These molecules can be considered as potential diagnostic and prognostic biomarkers, allowing response to therapy to be monitored.

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Keywords: miRNAs • PI3K/AKT/mTOR pathway • renal cell carcinoma

Renal cell carcinoma (RCC) is the most common renal malignancy, representing approximately 2% of all cancers, and it is the most lethal urological cancer. Kidney cancer accounts for approximately 84,000 new cases and 35,000 deaths in Europe every year. There is a 2:1 male predominance, with a peak incidence between 60 and 70 years [1].

RCC is a heterogeneous cancer which is divided into various subtypes, each derived from a different part of nephron with different genetic and molecular alterations, histological features, clinical phenotypes and prognosis. The major subtypes are the clear cell RCC (ccRCC), papillary RCC (pRCC) and chromophobe RCC (chRCC) [2]. The most common subtype of RCC is ccRCC, which accounts for approximately 80% of all cases, and it is characterized histologically by high cell lipid content and a richly vascularized tumor stroma [3]. ccRCC is the most aggressive subtype and it is associated with a high risk of metastasis formation, since most cases are not detected during the early stages [4]. Localized ccRCC can be treated with surgery, however, approximately 30% of these patients eventually develop metastases, which are associated with high mortality rates [1]. In consequence of the highly vascular nature, ccRCC rapidly becomes chemo- and radioresistant leading to a poor prognosis, thus requiring the development of new therapies or new therapeutic schemes [5].

Increasing molecular knowledge regarding the key signaling pathways deregulated in ccRCC has allowed, in recent years, the development of new target therapies [6]. The von Hippel–Lindau (VHL) pathway is one of the major signaling pathways involved in the pathophysiology of ccRCC. The protein encoded by the *VHL* gene, under normoxic conditions, serves as a recognition site for the regulatory subunits of HIF, targeting them with ubiquitin to proteasome degradation. In consequence of the alterations of *VHL*, due to the loss of the short arm of chromosome 3, or its inactivation or mutation, HIF degradation stops leading to its accumulation in the cytoplasm and migration to the nucleus. Here, it binds to hypoxia-related genes, resulting in a cell hypoxic response under normoxic conditions. These alterations lead to the transcriptional activation of genes involved in pathways responsible for angiogenesis and cell growth, such as the transcription of PDGF and VEGF [7].

Future Medicine

Another ccRCC oncogenic mechanism involves the activation of the mTOR pathway, a recurrent oncogenic event in cancer [8]. The mTOR protein is a highly conserved serine/threonine kinase that belongs to the PI3K-related kinase family. Through both intracellular and extracellular signaling, the mTOR pathway plays a role in the regulation of cell metabolism, growth, proliferation and survival [9]. mTOR is a downstream effector of the PI3K/AKT pathway and is also activated by genetic alterations that reduce the function of the tumor-suppressor PTEN or increase the function of the catalytic subunit of PI3K leading to abnormal activation of AKT. In addition, it has been elucidated that HIF protein expression is dependent of mTOR [10]. The discovery of the VEGF and mTOR involvement in ccRCC pathogenesis allowed the development of targeted therapies directed to these pathways [6].

There are two major types of targeted agents used in ccRCC treatment according to the key pathways deregulated: angiogenesis inhibitors that target the VEGF ligand or VEGF receptors (VEGFR; such as the multikinase inhibitors axitinib, sunitinib, pazopanib and sorafenib) and inhibitors of the mTOR signaling pathway (everolimus and temsirolimus) [2,11].

Additionally, cytokine therapies (IL-2 and IFN-α) constitute an interesting alternative to the standard metastatic ccRCC treatment. IFN-α and/or IL-2 that activate diverse immune effector cells have improved disease control rates and clinical outcomes. However, the significant toxicities of cytokines and the fact that they present less clinical efficacy when compared with tyrosine kinase inhibitors (TKIs) limits the use of these therapies [12]. Currently, targeted therapies have become the standard treatment for patients with metastatic RCC, replacing the cytokine therapies [13].

More recently, a new treatment approach has been developed, which modulates the immune system against tumor cells. This treatment targets the PD-1 receptor and PD-L1, leading to inhibition of the PD-1 checkpoint pathway. The development of targeted treatments has led to an improvement in RCC treatment outcomes, but fails to cure the disease mainly due to the development of resistance [14].

Additionally, several questions remain unanswered with regard to the optimal use of these agents, including the most effective sequence of targeted therapies, the benefits of targeted therapies in combination and how to overcome resistance development to these agents. It is important to note that, despite the prognosis improvement of metastatic ccRCC patients, the response to targeted therapies is varied and the majority of patients will eventually progress in the disease. Currently, no curative treatment for metastatic ccRCC is available [15].

Resistance to target therapies in RCC

The targeted agents approved for ccRCC treatment have increased progression-free survival and overall survival in the majority of patients [16]. However, these treatments have specific toxicity profiles, which can lead to dose reduction and even discontinuation of the treatment [17]. Additionally, the development of resistance also led to changes in dosing schedules in order to overcome the failures of first-line therapies and, currently, most of the therapeutic approaches include several lines of treatment per patient. Resistance is currently defined by the evidence of disease progression. This raises a clinical and scientific question regarding the mechanisms of resistance to TKIs and mTOR inhibitors [18]. In terms of therapy response, Ravaud *et al.* divided the patients into three groups. One subset of patients, approximately 25%, demonstrated resistance to therapy when they were initially assessed for response following 2–3 months of therapy. A larger group of patients showed initial tumor regression, followed by a short period of disease stability, and finally disease progression after 6–12 months of treatment. The third subset of patients experienced tumor regression in the first few months of therapy followed by a longer period of disease stability with no appearance of new lesions [19].

Drug resistance in ccRCC occurs to allow the survival of cancer cells and it is observed with the currently used targeted therapies such as VEGFR and mTOR inhibitors. The resistance to VEGFR inhibitors is often due to mutation in a gene encoding a key receptor tyrosine kinase. However, these mutations would have to take place in the tumor endothelium, which is the main target of VEGFR inhibitors and it is almost impossible that identical mutation coexists on each individual tumor metastasis. Another potential mechanism of resistance is upregulation of alternative proteins and/or pathways that re-establish angiogenesis and growth capacity in a VEGF-independent manner [18]. For example, upregulation of angiopoietin 2, c-MET or IL-8 signaling can trigger alternative angiogenesis, whereas the proliferation can be promoted through upregulation of the PI3K/AKT/mTOR pathway [8]. Other resistance mechanisms involve the occurrence of an increased drug efflux that results in decreased intracellular TKI concentrations in cells. TKIs are captured and stored in intracellular compartments instead of

reaching cancer cells promoting low concentrations in plasma and serum. Promotion of inflammation is another mechanism of resistance. Bone marrow-derived cells modulate expression of a wide variety of cytokines, growth factors and enzymes promoting tumor adaptation and resistance to targeted therapies [20].

mTOR inhibitors form a complex with the FK binding protein and inhibit mTOR from binding complex 1 (mTORC1). However, the other mTOR complex, mTOR complex 2 (mTORC2) may not be inhibited by these targeted therapies. These facts suggest that inhibition of mTORC1 leads to a compensatory activation of PI3K/AKT pathway via upregulation of mTORC2. mTORC2 activates AKT and HIF-2α, limiting the effect of these therapies [14].

This suggests that further investigation of the molecular pathology of RCC is needed which may then lead to the identification of potential biomarkers that are predictive of tumor sensitivity to PI3K/AKT/mTOR-targeted therapies. It is also important to identify new targets in order to develop new treatments. Several studies described the involvement of miRNAs dysregulation in the pathogenesis and progression of ccRCC [21]. The cellular effects of miRNAs dysregulation are diverse and often lead to typical hallmarks of cancer. These small molecules can be considered as potential diagnostic and prognostic biomarkers, allowing the monitoring of disease, as well as promising new therapeutic agents [14].

miRNAs

miRNAs are a class of short ncRNA molecules (~22 nucleotides of length) that regulate gene expression at the post-transcriptional level, through binding to the complementary region of corresponding mRNAs targets, leading to the inhibition or degradation of mRNAs [22]. Most primary transcripts of miRNAs are produced in the nucleus. Primary miRNAs (pri-miRNA) are transcribed by RNA polymerase II and are processed by Drosha and its cofactor DGCR8, creating a pre-miRNA. The pre-miRNAs are carried out to the cytoplasm by the XPO5, where it is cleaved by Dicer to generate the mature double-stranded miRNA. The mature miRNAs are incorporated into the RNA-induced silencing complex, which will guide them to the complementary region of their targets and this process results in the inhibition of mRNA translation, or promotes its degradation and leads to post-transcriptional gene silencing [23].

Recent publications suggest that miRNAs have an important role in cancer development by influencing all cancer hallmarks, which include: resistance to cell death, genome instability and mutation, induction of angiogenesis, activation of invasion and metastasis, tumor-promoting inflammation, replicative immortality capacity, avoid to the immune destruction, evading growth suppressors, sustaining proliferative signaling and deregulating cellular energetics [24].

miRNAs may have more than one target while different miRNAs can interact with the same mRNA. Several studies have shown that miRNAs are expressed or inhibited in different types of cancer, suggesting that miRNAs work as onco-miRNA or tumor-suppressor miRNA. Through miRNA expression profiling analysis onco-miRNAs which downregulated tumor suppressor genes and are overexpressed in cancer cells were identified [23]. For example, Yu *et al.* showed that miR-7 expression increases significantly in RCC compared with normal adjacent tissues and miR-7 serum levels from RCC patients were higher than in healthy individuals [25]. Other studies identified tumor-suppressor miRNAs that are underexpressed in cancer. Zhu *et al.* compared miR-451 levels in RCC tissues and in the corresponding adjacent normal tissues and showed that miR-451 levels in RCC tissues were lower than in normal tissues [26]. Recent studies identified a number of miRNAs that are deregulated in ccRCC, which can be used as potential biomarkers for early cancer diagnosis and prognosis, as well as potential targets for more efficient treatments [23,27].

The circulating levels of specific miRNAs are promising noninvasive blood-based biomarkers with a high potential for the early diagnosis of RCC and monitoring of therapy response since they may be predictive of resistance therapies. According to a previous publication from our group, the increased miR-221 levels play a key role in cellular microenvironment, modulating important cellular processes involved in carcinogenesis and cancer progression. We also observed that RCC patients and healthy individuals have different expression levels of circulating miR-221 [28].

Recent research data suggest that miRNAs may be also related to resistance to conventional therapies. Goto *et al.* demonstrated that miR-101 exhibits antitumor activity and is significantly suppressed in sunitinib-treated RCC tissues compared with the primary RCC tissues [29].

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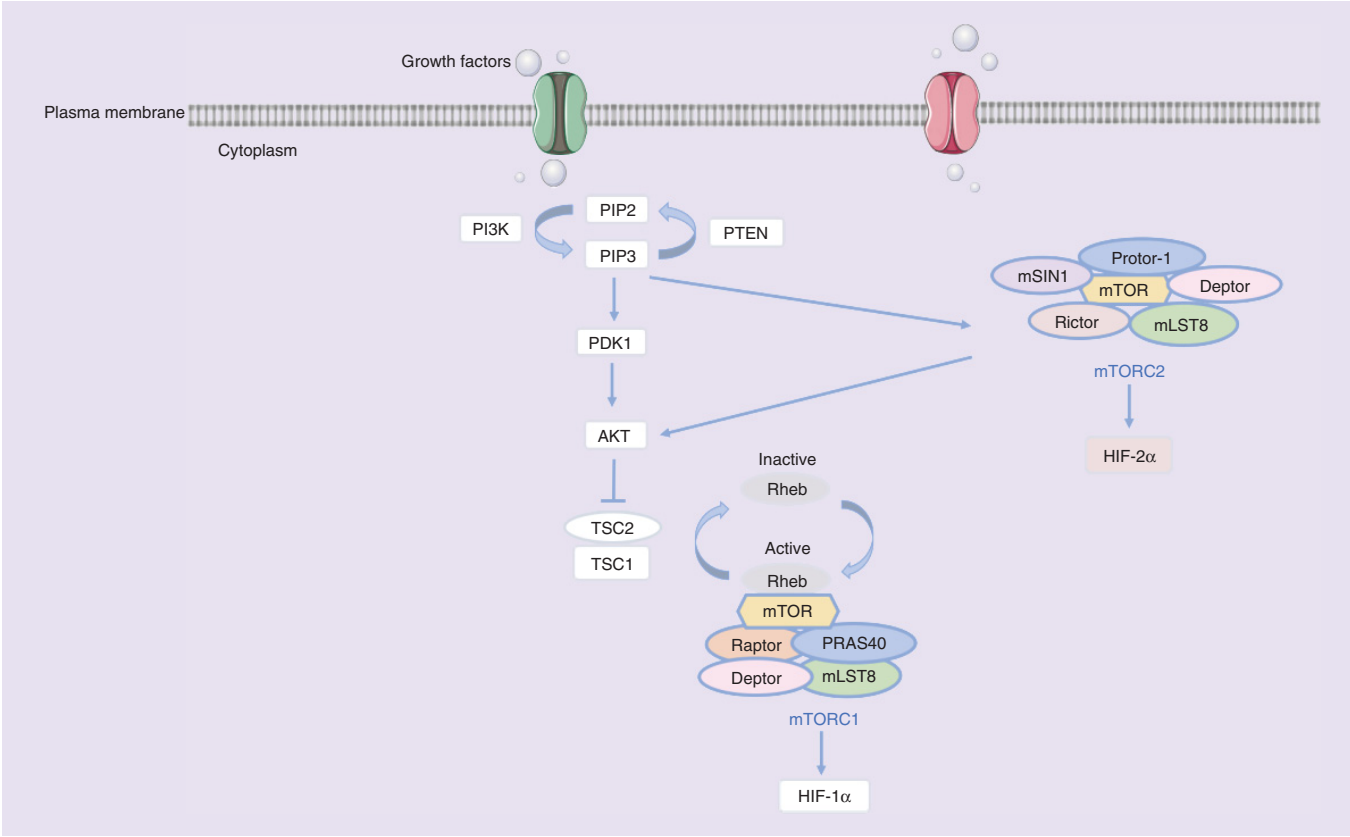

Figure 1. Schematic representation of the miRNAs that are involved in the regulation of PI3K/AKT/mTOR pathway in cancer.

PI3K/AKT/mTOR pathway

The PI3K pathway is a signal transduction cascade that is responsible for many physiological functions, including cell cycle, cell survival, protein synthesis and growth, metabolism, motility and angiogenesis (Figure 1). The PI3K family is divided into four different classes: class I, class II, class III and class IV. Class I are heterodimeric molecules composed by a regulatory subunit: PIK3R1, PIK3R2 and PIK3R3 and a catalytic subunit: PIK3CA, PIK3CB and PIK3CD. The class IV is composed by ATM, DNA-PKcs and mTOR [30]. PI3K converts its substrate phosphatidylinositol 4,5-biphosphate–PI (4,5) P2 into PI (3,4,5) P3 (or PIP3). However, PTEN antagonizes PI3K activity and negatively regulates AKT signaling. Protein kinase B, also known as AKT, is a serine/threonine-specific protein kinase, which is involved in multiple cellular processes. AKT can appear in one of three isoforms (AKT1, AKT2, AKT3) [9].

PDK1 is recruited to the membrane and phosphorylates AKT at Ser308 and mTORC2 phosphorylates AKT at Ser473 promoting full activation of AKT. AKT inhibits through phosphorylation TSC1/2. TSC1/2 functions as a GAP and that way negatively regulates mTORC1 signaling by converting Rheb into its inactive form. The active form of Rheb directly interacts with mTORC1 to stimulate its activity [31].

mTOR acts as a downstream effector of the PI3K/AKT pathway and is the catalytic subunit of the two biochemically distinct complexes, mTORC1 and mTORC2. mTORC1 has five components: mTOR, which is the catalytic subunit of the complex; rRaptor; mLST8 (also known GβL); PRAS40 and Deptor. mTORC2 comprises six different proteins, of which some are common to mTORC1: mTOR; Rictor; mSIN1, Protor-1; mLST8 and Deptor [10]. Each complex has a distinct protein composition, reflecting differences in upstream signal integration, substrate regulation and biological process control. mTORC1 is regulated by nutrients, growth factors, cellular energy and stress pathways, while mTORC2 is primarily regulated by growth factors. mTORC1/2 are both positively regulated by IFNs. The mTOR complexes have distinct functions. mTORC1 has a positive regulatory effect on cell growth and proliferation through the promotion of many anabolic processes, including biosynthesis of proteins, lipids and organelles and by limiting catabolic processes such as autophagy. mTORC2 plays key roles

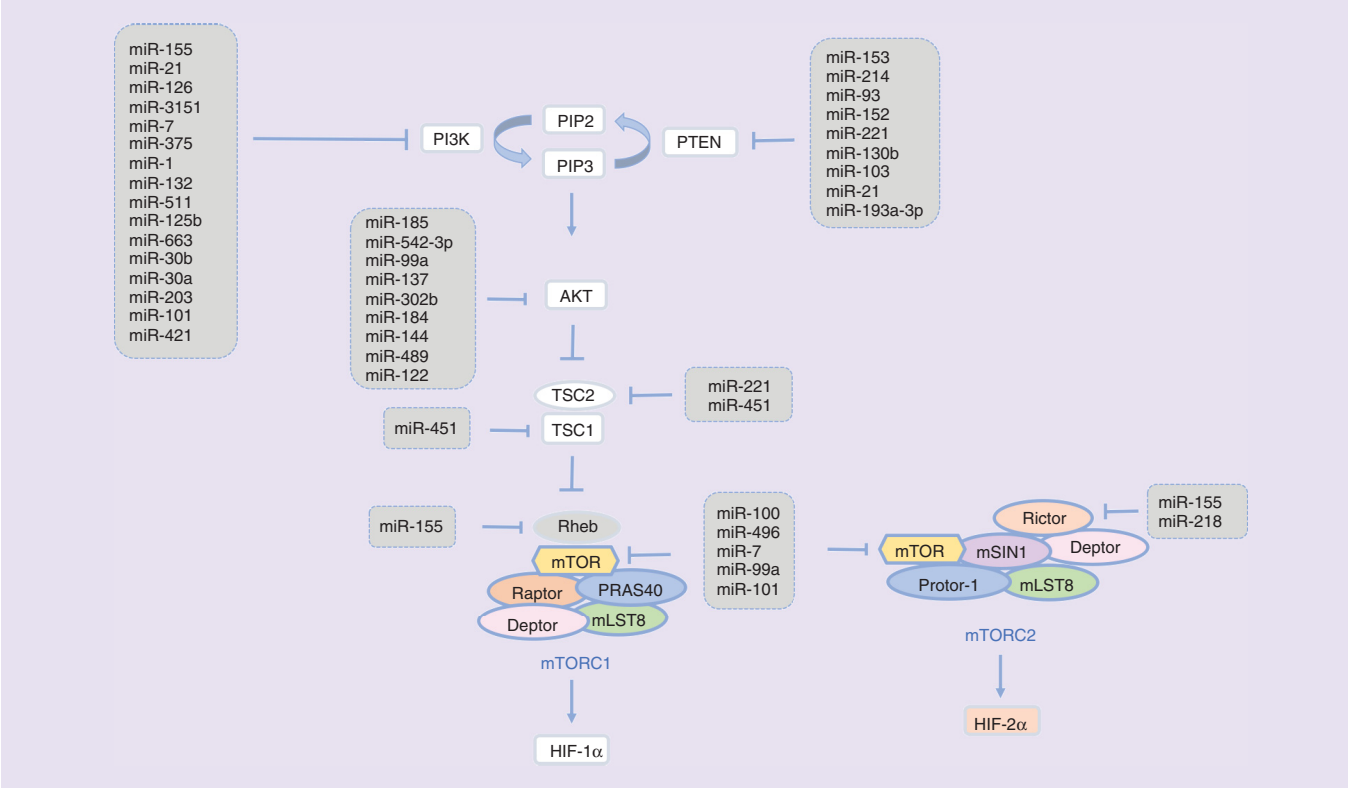

Figure 2. Schematic representation of the miRNAs that are involved in the regulation of PI3K/AKT/mTOR pathway in cancer.

in various biological processes, including cell survival, metabolism, proliferation and cytoskeleton organization. mTOR signaling is often deregulated in ccRCC, which can lead to an upregulation of HIF and a worse prognosis for the patients [9].

miRNAs & PI3/AKT/mTOR pathway in cancer

The PI3K/AKT/mTOR pathway, a vital signal transduction system-linking oncogenes and multiple receptor classes to several essential cellular functions, is perhaps the most commonly activated signaling pathway in human cancer. Nowadays, it is known that miRNAs have an important role in regulation of mTOR signaling in most cancer types.

In Figure 2, we summarize all the currently known miRNAs, with regulatory functions in the PI3K/AKT/mTOR pathway that were described as deregulated in cancer.

Evidence acquisition

A systematic literature search of PubMed was conducted using the following keywords: miRNAs, PIK3R1, PIK3R3, PIK3CA, PIK3CD, ATM, DNA-PKcs, PTEN, TSC1, TSC2, mTOR, AKT1, AKT2, AKT3, Rheb, Rictor. The articles were selected by relevance of their findings. All the references of the cited papers were reviewed and relevant publications in the field of PI3K/AKT/mTOR signaling involving the studied molecules were added.

Evidence synthesis

Investigations have shown that PIK3R1 (p85α), as negative regulator of the PI3K/AKT pathway, is a direct target of miR-155 and miR-21. In fact, Huang *et al.* found that, in diffuse large B-cell lymphoma cell lines and in blood samples, the overexpression of miR-155 leads to the downregulation of PIK3R1-activating AKT signaling [32]. Studies on breast and pancreatic cancers also showed that miR-21 and PIK3R1 expression are inversely correlated promoting cell growth, migration and invasion [33,34].

Recent findings have shown that miR-3151 inhibits the PI3K/AKT pathway by repressing PIK3R2 in chronic lymphocytic leukemia-inhibiting cell proliferation and enhancing apoptosis [35]. Studies in esophageal squamous cell

carcinoma and bladder cancer have demonstrated that PIK3R2 is a direct target of miR-126-inhibiting proliferation, migration, invasion and promoting apoptosis [36,37].

Other studies have shown that PIK3R3 is a target gene of miR-132 and miR-511. Overexpression of these two miRNAs inhibits PIK3R3 expression and PI3K/AKT signaling activation. These findings showed that miR-132 and miR-511 function as tumor suppressors in hepatocellular carcinoma. These miRNAs can inhibit cell proliferation, migration and invasion and induced cell apoptosis in hepatocellular carcinoma [38,39].

Yu *et al.* showed that miR-1 is a potential tumor suppressor by targeting PIK3CA in non-small-cell lung cancer (NSCLC) cell lines. Its repression of PIK3CA expression may play an important role in NSCLC progression [40]. Studies in human colorectal cancer and osteosarcoma identified miR-375 as a tumor growth suppressor through repression of PI3K/AKT pathway by inhibiting PIK3CA [41,42].

Studies have shown that PIK3CD is a direct target of several miRNAs. Fang *et al.* showed that miR-7 regulates cell proliferation and metastasis formation through the PI3K/AKT pathway and these findings were observed in hepatocellular carcinoma [43]. Studies in colorectal cancer demonstrated that PIK3CD is a direct target of miR-30a and miR-30b and may affect tumor cell survival-inhibiting cell migration and invasion [44,45]. Shi *et al.* showed that miRNA-663 inhibited the proliferation and invasion of glioblastoma cells *in vitro* and *in vivo* by directly targeting PIK3CD [46]. Various studies demonstrated that miR-125b acted as a tumor suppressor through suppression of the PI3K/AKT signaling pathway by targeting the *PIK3CD* gene. These findings were observed in anaplastic thyroid cancer, cervical cancer and Ewing's sarcoma [47–49].

Recent findings have shown that ATM is a direct target of miR-203, miR-101 and miR-421 [50–52]. Studies in colorectal cancer showed that miR-203 negatively regulates ATM by binding to a conserved site of the ATM 3′-UTR-inducing cell growth delay and senescence and investigations showed that miR-203 and ATM have an important role in inducing an acquired chemoresistant phenotype in CRC cells [50]. Overexpression of miR-101 inhibits the expression of DNA-PKcs and ATM in lung cancer and glioma cells and thus sensitizes tumors to radiation [51]. Studies in neuroblastoma showed that miR-421 and ATM expression are inversely correlated inducing tumorigenesis in neuroblastoma [52].

Investigations have shown that PTEN is a direct target of miR-153, miR-214, miR-93, miR-152, miR-221, miR-130b and miR-103. These miRNAs act as oncomiRs promoting prostate cancer, breast cancer, hepatocellular carcinoma, nasopharyngeal carcinoma, cervical cancer, esophageal squamous carcinoma and colorectal cancer development, respectively [53–59]. Moreover, recent studies showed that miR-21 and miR-193a-3p targets PTEN in a several types of cancers promoting uncontrolled growth, metabolism and metastasis [60].

Du *et al.* identified TSC1 as a direct target of miR-451. These findings were observed in myeloma cell lines and *in vivo*. Thus, miR-451 mediate PI3K/AKT/mTOR pathway that plays an essential role in myeloma stem cell biology [61]. Wan *et al.* showed that miR-155 could suppress the activation of mTORC1 and AKT through the inhibition of Rheb and Rictor in nasopharyngeal carcinoma and cervical cancer cell lines. miR-155 attenuates cell proliferation and induces G_1/S cell cycle arrest [62]. Investigations in oral cancer, medulloblastoma and prostate cancer demonstrated that miR-218 acts as a tumor suppressor by directly binding to Rictor-inhibiting tumorigenesis [63–65]. Investigations have shown that TSC2 is a direct target of miR-221 in pancreatic cancer, thyroid papillary carcinoma, breast cancer, glioblastoma, NSCLC, small-cell lung cancer and hepatic cellular cancer. On the contrary, miR-451 targets TSC2 in glioma. These miRNAs promote tumor development [60].

Several studies showed that mTOR is a direct target of various miRNAs. Lin *et al.* demonstrated that miR-101 is a tumor-suppressor gene, often found to be downregulated in osteosarcoma. Overexpression of miR-101 inhibits osteosarcoma cell proliferation and promotes apoptosis by targeting mTOR [66]. Investigations showed that miR-99a plays an important role in breast cancer by directly targeting mTOR and reversing the breast cancer malignant phenotype [67]. Recent studies demonstrated that miR-7 and miR-496 inhibit mTOR. These findings were observed in hepatocellular carcinoma and cervical cancer, respectively [43,68]. Investigations in bladder urothelial cancer and chondrosarcoma showed mTOR as a direct target of miR-100. These miRNAs act as tumor suppressor by inhibiting PI3K/AKT/mTOR signaling [69,70].

Several studies demonstrated that AKT1 is a direct target of miR-185, miR-99a and miR-542–3p and these findings were observed in lung cancer and astrocytoma. These facts suggest that proliferation, migration and invasion are regulated by these miRNAs [71–73].

Recent findings showed that miR-137, miR-302b and miR-184 regulate PI3K/AKT signaling through the direct inhibition of AKT2 in gastric cancer, hepatocellular carcinoma and neuroblastoma studies, respectively [74–76].

Table 1. miRNAs that are deregulated in renal cell carcinoma and target different components of PI3/AKT/mTOR pathway.

Up-/downregulation	miRNAs	Function	Target genes	Tissues/cell lines/serum/*in vivo*	Subtype	Ref.
↗	miR-92	OncomiR	VHL	Tissues/cell lines	ccRCC; papillary RCC and chromophobe RCC	[81]
↗	miR-21	OncomiR	PTEN	Tissues/cell lines	–	[79,80]
↙	miR-22	OncomiR	PTEN	Tissues/cell lines	ccRCC	[82]
↗	miR-23b-3p	OncomiR	PTEN	Tissues/cell lines	–	[83]
↙	miR-99a	Tumor suppressor	mTOR	Tissues/cell lines/nude mice	–	[84]
↙	miR-144	Tumor suppressor	MTOR	Tissues/cell lines	–	[85]
↙	miR-137	Tumor suppressor	Predicted target: PI3K/AKT	Tissues/cell lines/BALB/c mice	–	[86]
↙	miR-182–5p	Tumor suppressor	HIF-2α	Tissues/cell lines/BALB/c nude mice	ccRCC	[87]
↙	miR-101	Tumor suppressor	DNA-PKcs	Tissues/cell lines/nude/beige mice	–	[88]
↙	miR-148a	Tumor suppressor	AKT2	Tissues/cell lines	ccRCC	[89]

AKT: Protein kinase B; ccRCC: Clear cell renal cell carcinoma; DNA-PKcs: DNA-dependent protein kinase; HIF: Hypoxia-inducible factor; PI3K: Phosphoinositide 3-kinase; VHL: von Hippel–Lindau pathway.

Studies in hepatocellular carcinoma showed that AKT3 is a direct target of miR-144 and miR-122 [77,78]. Wu *et al.* demonstrated that miR-489 negatively regulate AKT3 expression by direct binding sites in its 3′-UTR. Thus, these miRNAs inhibit cell growth [79].

miRNAs & PI3K/AKT/mTOR pathway in RCC

In this section, we will focus on the functional role of miRNAs in the regulation of PI3K/AKT/mTOR pathway in RCC. A comprehensive list of miRNAs targeting this pathway is listed in Table 1.

miRNA-21 is involved in cellular mechanisms such as cell growth, apoptosis, cell cycle, invasion and migration of tumor cells and this miRNA inhibits *PTEN*, a tumor suppressor gene. Therefore, significantly higher miR-21 levels were associated with higher stage and tumors grade and these results were demonstrated in RCC tissues and cell lines [80,81]. Studies in samples of RCC tissues and cell lines showed that a higher expression of miR-92 may be related to a more aggressive phenotype by inhibiting the *VHL* expression [82].

Fan *et al.* showed that miR-22 is downregulated in ccRCC and presents the ability to inhibit cell growth, migration and invasion by directly targeting PTEN. These findings were demonstrated in tumor tissues and cell lines [83]. Investigations in RCC tissues and cell lines demonstrated that miR-23b-3p is an oncogene and directly inhibits the *PTEN* tumor-suppressor gene [84].

Recent findings have shown that miR-99a, miR-137, miR-182–5p and miR-101 were downregulated in RCC. Cui *et al.* identified in patient's tissues and cell lines the miR-99a as a potential tumor suppressor by inhibiting mTOR and these results were also demonstrated *in vivo* [85]. Recent studies showed in patient tissue samples and cell lines that miR-144 inhibits cell proliferation of RCC by directly targeting mTOR [86].

Zhang and Li showed in patient tissues, cell lines and *in vivo* xenograft models that miR-137 acts as a tumor suppressor by inhibiting the PI3K/AKT pathway [87].

Downregulation of miR-182–5p plays an important role in the pathogenesis of RCC. Studies demonstrate that this miRNA functions as a tumor suppressor, by inhibiting HIF-2α, a promoter of tumor growth and angiogenesis. These results were consistent with the *in vivo* findings [88].

Zheng *et al.* showed that miR-101 level was significantly lower in human RCC tissues and cell lines and inhibits indirectly the mTOR pathway by targeting DNA-PKcs, a member of PI3K, that regulates mTOR activation. Studies in xenograft models *in vivo* showed that DNA-PKcs inhibition or silencing supresses AKT phosphorylation, HIF-2α expression and tumor growth [89]. Recent investigations in RCC tissues and cell lines demonstrated that AKT2 is a direct target of miR-148a [90].

In summary, miRNAs have been clearly demonstrated to have relationships with mTOR signaling pathway in RCC. Expression profiling of miRNAs has been used to understand the development, invasion and progression of cancer since they interfere with many different cellular processes and they can act as a tumor suppressor or oncogenes to promote uncontrolled growth, metabolism and metastasis.

The involvement of miRNAs in the pathogenesis of RCC, describing their potential as novel diagnostic and prognostic biomarkers, as well as predictive biomarkers for therapeutic response [91].

Because mTOR is a validated therapeutic target for cancer, and the clinical practice is facing some problems due to the development of resistance to anti-mTOR therapies, targeting these miRNAs may provide a novel approach to facilitate an integrated anticancer therapy [92].

Two therapeutic strategies may be applied using miRNAs: inhibition or replacement. Through the delivery of antagomiRs, we are able to silence endogenous oncomiRs, therefore inhibiting their action on tumor-suppressor mRNAs. On the other hand, through the ectopic replacement of tumor-suppressive miRNAs, by delivery primary miRNA or miRNA synthetic mimics, we can restore tumor-suppressive miRNAs levels. Both these mechanisms of miRNA delivery can be made in several ways, such as inside exosomes, which allow a targeted approach with minimal side effects for the patients [93].

The efficacy and safety of miRNA-derived drugs must be carefully assessed and it will depend on the tumor cells type and context [94]. Moreover, circulating miRNAs may be captured for liquid biopsy, since alterations in their expression levels can be related to prognosis and therapy response prediction. The specificity and sensitivity of mTOR inhibitors may be further improved by using these new therapeutic approaches [60].

Conclusion & future perspective

In this review, we highlighted an altered pathway in RCC that may be further studied to improve diagnosis and prognosis as well as the development of new therapeutic strategies. PI3K/AKT/mTOR pathway is altered in the majority of cancer cells-promoting tumorigenesis. This pathway is induced as a consequence of the one of the first molecular events associated with RCC, and can be modulated by several miRNAs.

Since these miRNAs seem to be deregulated in cancer, one new therapeutic approach could be restoring suppressor miRNAs levels or the inhibition of oncogenic miRNAs, which would inhibit mTOR signaling and, consequently, one of the major deregulated pathways in tumorigenesis. Further studies are necessary to demonstrate and validate the application of the previously described miRNAs as therapeutic candidates for RCC management.

In the future, the knowledge of the expression profile of mTOR-related miRNAs may contribute to the monitorization of targeted therapies response in order to predict the resistance to mTOR therapies and, consequently, improve the patients' follow-up and care.

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Executive summary

Renal cell carcinoma

- Renal cell carcinoma (RCC) is the most common renal malignancy, and the most lethal urological cancer.
- RCC is a heterogeneous cancer which is divided into various subtypes. The most common subtype of RCC is clear cell RCC (ccRCC), accounting for approximately 80% of all cases.
- One of the signaling pathways involved in the pathophysiology of ccRCC is the von Hippel–Lindau pathway. In consequence of the alterations of von Hippel–Lindau pathway, the degradation of hypoxia-inducible factor stops, which leads to its accumulation in the cytoplasm and further migration to the nucleus, where it binds to hypoxia-related genes.
- Another ccRCC oncogenic mechanism involves the activation of the mTOR pathway. The mTOR signaling serves as a central regulator of cell metabolism, growth, proliferation and survival.
- The discovery of these pathways in ccRCC pathogenesis allowed the development of targeted therapies: angiogenesis inhibitors (multikinase inhibitors) and mTOR inhibitors.

Resistance to target therapies in RCC

- Drug resistance in ccRCC occurs to enable cancer cells survival and it is present in the currently used targeted therapies, such as VEGF and mTOR inhibitors.
- These facts point to an urgent need to make further investigations of the molecular pathology of ccRCC and identification of potential biomarkers that are predictive of tumor sensitivity to mTOR-targeted therapies.

miRNAs & cancer

- miRNAs are a family of small noncoding RNAs that regulate gene expression at the post-transcriptional level, by binding to the complementary region of corresponding mRNAs leading to the inhibition or degradation of mRNAs.
- miRNAs are expressed or inhibited in different types of cancer, suggesting that miRNAs work as oncomiRNAs or tumor-suppressor miRNAs.
- These small molecules can be considered as potential diagnostic and prognostic biomarkers, as well as predictive biomarkers for therapeutic response allowing disease monitorization.
- The circulating levels of specific miRNAs are promising noninvasive blood-based biomarkers.

miRNAs & PI3/AKT/mTOR pathway

- miRNAs have an important role in regulation of mTOR signaling in most cancer types, including RCC.
- miR-101 level was significantly lower in human RCC tissues and cell lines and inhibits indirectly the mTOR pathway by targeting DNA-dependent protein kinase, and that regulates mTOR activation.
- Two therapeutic strategies may be applied using miRNAs in order to reverse mTOR therapy resistance: through the delivery of antagomiRs in order to silence endogenous oncomiRs, and through ectopic replacement of tumor-suppressive miRNAs by delivery primary miRNA or miRNA synthetic mimics.

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