ONCOLOGY/RECONSTRUCTION

REVIEW

Prognostic markers in renal cell carcinoma: A focus on the 'mammalian target of rapamycin' pathway

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Abstract   *Objectives:* Increased knowledge about the molecular pathways involved in tumorigenesis has led to the discovery of new prognostic molecular markers and development of novel targeted therapies for renal cell carcinoma (RCC). In this review we describe the prognostic markers of RCC and highlight the areas of recent discovery with a focus on the mammalian target of rapamycin (mTOR) pathway.

Methods: We reviewed previous reports, using PubMed with the search terms 'renal cell carcinoma', 'molecular markers', 'prognosis', 'outcomes' and 'mammalian target of rapamycin pathway' published in the last two decades. We created a library of 100 references and focused on presenting the recent advances in the field.

Results: Growing evidence suggests that mTOR deregulation is associated with many types of human cancer, including RCC. Consequently, temsirolimus and everolimus, which target mTOR, are approved for treating advanced RCC. There is a demand to integrate clinical, pathological and molecular markers into accurate prognostic models to provide patients with the most personalised cancer care possible.

Conclusions: The mTOR pathway is highly implicated in RCC tumorigenesis and progression, and its constituents might represent a promising prognostic tool and target for treating RCC. Combining newly discovered molecular markers with

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S6K1, S6 kinase 1; 4E-BP1, eukaryotic initiation factor-binding protein-1; TKR, tyrosine kinase receptor; TSC, tuberous sclerosis complex; IRS-1, insulin receptor substrate-1; CA-9, carbonic anhydrase 9

classic clinicopathological prognostics might potentially improve the management of RCC.

Introduction

RCC accounts for ≈3% of all cancer diagnoses [1]; while the classic clinicopathological tools, such as tumour stage and grade, are apparent at the time of diagnosis using modern imaging and pathology, they are still inadequate in predicting the prognosis of RCC. Advances in molecular biology and genetics have provided an insight into the detailed molecular alterations and subsequent downstream pathways involved in tumorigenesis and disease progression. Understanding and knowing more about tumour biology are extremely important to improve the ability to predict the outcome and response to systemic therapies, especially in the era of targeted therapies [2]. With the development of these novel targeted therapeutics, the conventional well-established prognostic models based on clinical and pathological variables might not be the best tools for clinical use. The integration of molecular markers into prognostic and predictive models will change the management paradigms. Eventually, it will allow tailoring of multimodal treatments, with a choice of different medical and surgical options for individuals in the present era of personalised medicine.

The mammalian target of rapamycin (mTOR) is a key regulator of cell growth and proliferation. It regulates essential signal-transduction pathways and is involved in coupling growth stimuli to cell-cycle progression, and thus alterations in its function are highly implicated in carcinogenesis [3]. It has recently received special interest in RCC because of the development of such targeted therapeutic agents everolimus and temsirolimus, which are approved for treating patients with advanced RCC. Our goal in this review was to provide a broad overview of the current state of prognostic markers in RCC (summarised in Table 1), specifically the constituents of mTOR pathway, to establish a basis for understanding their future utility.

Pathologic markers

TNM staging is still the most important pathological prognostic marker and is periodically subjected to updates. The TNM classification for RCC has been recently updated (2009) [4]. There have been alterations to the system accounting for tumour size and patterns of regional involvement, such as peri-renal fat, renal sinus and renal vein invasion [4]. Also, investigation into variations in nodal involvement, such as extra-nodal tumour extension and lymph-node density, has been shown to affect the prognosis [5,6]. However, despite the recent revision, or even with future changes, there will still be questions about the validity and prognostic ability of this system alone [7,8].

Clinical markers

Laboratory tests like haemoglobin level, platelet count, serum calcium, erythrocyte sedimentation rate, C-reactive protein, lactate dehydrogenase (LDH) and serum ferritin have been correlated with outcomes in RCC. A recent meta-analysis found C-reactive protein, platelet count and erythrocyte sedimentation rate to be the independent predictors of relapse-free and cancer-specific survival [9]. The role of platelets in tumorigenesis might be related to the fact that they harbour vascular endothelial growth factor (VEGF) and other tumour-promoting factors. Performance status was commonly used to predict those who benefit most from cytoreductive nephrectomy [10,11]. However, clinical markers are

Table 1	Prognostic factors in RCC.
Type	Factors
Pathological	TNM staging, newly developed nodal prognostic factors: extra-nodal tumour extension and lymph node density
Clinical	Performance status and absence of previous nephrectomy
Laboratory	Haemoglobin level, platelet count, serum calcium, erythrocyte sedimentation rate, C-reactive protein, LDH and serum ferritin
Molecular	mTOR pathway: PI3K, Akt, pS6K, 4-E-BP1 VHL/HIF pathway: HIF-1α, VEGF, CA-9 Cell cycle markers: p53, p21, p27, Ki-67 Apoptotic markers: Bcl-2, APAF1, Survivin

insufficient as prognostic tools when used alone. Clinical markers are not specific for the tumour biology and merely reflect the host response or level of tumour burden. Their level can be altered by many factors, like improved nutrition or targeted therapeutics [12,13].

Inadequacy of clinicopathological variables in the prognosis of RCC

Combining clinical and pathological variables in a risk-stratifying model might help more as a prognostic tool. However, these variables still fail to completely capture the contribution of tumour biology to the patient's prognosis. The Memorial Sloan Kettering Cancer Center RCC risk classification, also known as the Motzer Criteria [14] identified low performance status, high serum LDH, low haemoglobin, high serum calcium and absence of previous nephrectomy as prognostic of poor survival rates in patients with metastatic RCC. It was established in the immunotherapy era, and with the quickly changing landscape in RCC, the development of novel targeted therapeutics and their relationship to changing surgical paradigms, the role of such markers must be continually reassessed. Molecular alterations should be potentially considered in prognostic calculations; especially, they might also help in predicting the response to targeted therapies. However, no markers, including those related to mTOR, are yet used clinically for prognostication in RCC.

Molecular markers

Combining molecular alterations reflecting the tumour's biology with the clinical and pathological factors will more accurately determine the prognosis. The growing body of knowledge related to RCC molecular pathways and molecular alterations has tremendously changed the clinical management of RCC. The mTOR pathway, von Hippel-Lindau (VHL)/hypoxia inducible factor (HIF) pathway, cell-cycle regulators and apoptotic markers are extensively studied in the era of targeted therapies.
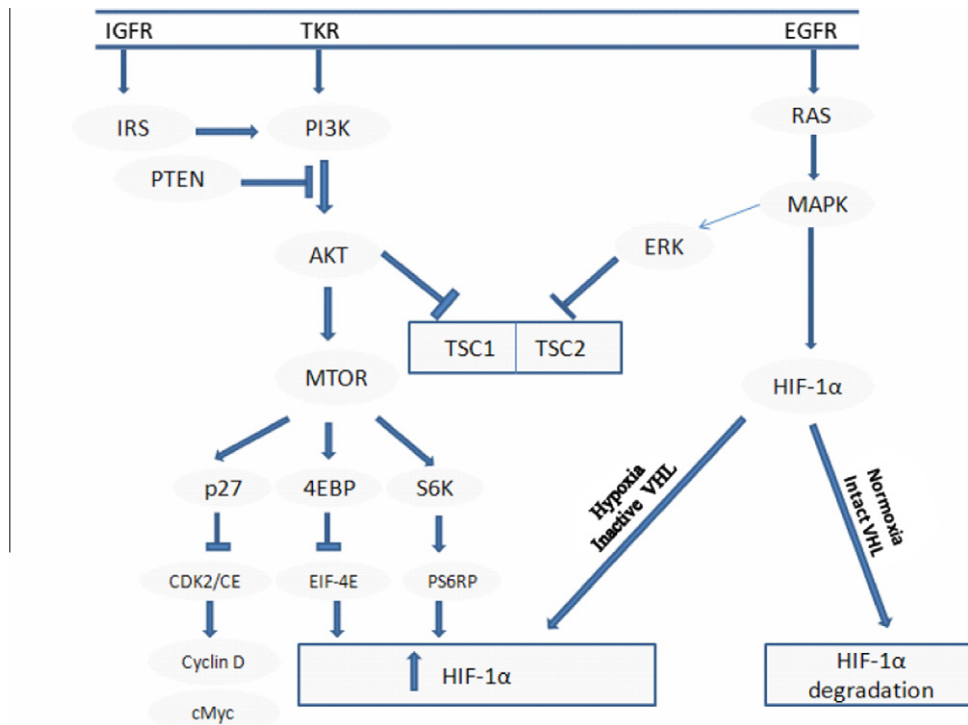
The mTOR pathway

mTOR is a high molecular weight serine-threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3k)-related kinase super-family. It acts as a central regulator for cell growth, proliferation, survival and angiogenesis, and thus alterations in its function are highly implicated in carcinogenesis [3]. It has recently received special interest in RCC because of the development of targeted therapeutics like temsirolimus and everolimus, both approved for treating advanced RCC. There are two distinct mTOR complexes, i.e. mTORC1 and mTORC2. The former phosphorylates

S6 kinase 1 (S6K1) and eukaryotic initiation factor-binding protein (4E-BP1), while mTORC2 controls the actin cytoskeleton and regulates the activity of AKT/protein kinase B, a phosphorylation pathway well described in carcinogenesis [15].

Fig. 1 illustrates the mTOR pathway and its relation to many cellular process and pathways. Growth factors like IGF, epidermal growth factor, platelet-derived growth factor and VEGF bind to and activate tyrosine kinase receptors (TKRs). Activated TKRs induce intracellular signalling cascades via PI3K/Akt, which in turn activates many cellular processes, including cell growth, proliferation, survival, angiogenesis and metabolism. TKRs also activate Ras, which not only activates the mitogen activated protein kinase pathway, but also activates PI3K, leading to the activation of mTOR [16]. Activated Akt inhibits tuberous sclerosis complex TSC1 and TSC2, the natural inhibitors of mTOR [17]. PTEN, a tumour-suppressor gene located on chromosome 10, antagonises PI3K function, negatively regulating Akt/mTOR activity and ultimately terminating the intracellular PI3K signalling cascade [18]. PTEN is absent or decreased in many cancers, and its deletion is associated with the metastatic disease in RCC [19]. Cells deficient in PTEN show high activity of the Akt/mTOR survival pathway, which makes them resistant to apoptosis, with a potential contribution to therapeutic resistance.

The phosphorylated mTORC1 complex controls cellular replication through S6K1 and 4EBP1. Typically, unphosphorylated 4E-BP1 inhibits the initiation of protein translation. However, after phosphorylation by mTOR, 4E-BP1 dissociates from eIF-4E, ultimately increasing the synthesis of HIF-1α and angiogenic factors like VEGF and fibroblast growth factor, thus connecting the mTOR pathway to angiogenesis and cell survival [3,20]. Cyclin D1 and c-myc are also among the downstream effectors of the mTOR pathway, explaining its role in cell proliferation [3], and mTOR activity might be considered a major gatekeeper for cell-cycle progression [21]. Phosphorylation of another downstream target of mTORC1, S6K1, leads to another path towards cell-cycle progression [22]. The phosphorylation status of S6K1 or 4E-BP1 is often used as a measure of mTOR activity in laboratory studies, which might be of value in predicting the therapeutic benefit of mTOR inhibitors in the clinic [3]. The second downstream target is S6K1, which phosphorylates the 40S ribosomal protein S6, enhancing the translation of mRNAs with a 5-terminal oligopyrimidine tract (TOP mRNAs), such as elongation factor-1 and ribosomal proteins [23]. S6K1 is implicated in protein synthesis, cytoskeletal rearrangement, splicing, cell survival and feedback regulation of multiple pathways, including mTOR [24]. S6K downregulates insulin signalling via phosphorylation and proteasomal degradation of

Figure 1 The mTOR pathway and its interactions with other important pathways and cellular process relevant to cancer.

insulin receptor substrate-1 (IRS-1) on prolonged mTOR activation. Inactivation of IRS-1 quenches IGF-1R from activating the PI3K/Akt/mTOR pathway, which is reversed by prolonged mTOR inhibition [25]. This negative feedback might account for the resistance of tumours to mTOR inhibitors. These drugs, while suppressing the downstream functions of mTOR signalling pathway, subsequently release the inhibitory signal of S6K1 on IRS-1. This leads to a paradoxical activation of Akt pathways that could lead to tumour progression. S6K1 has been investigated as a marker, and correlated with increased RCC stage and grade, and decreased disease-specific survival [26], and low S6K1 expression was linked to an increased therapeutic response [27]. Meanwhile, levels of PTEN and HIF-1α failed to correlate with the response to systemic therapy or oncological outcomes in a recent randomised phase III study [28].

Activation of the mTOR pathway also regulates bioenergetics, nutrient use and metabolism [29].

mTOR activation supports cellular growth and survival by increasing access to nutrients and metabolic fuels via biosynthesis (translation) of nutrient transporter proteins like LAT1 for amino acids, GLUT1 for glucose and transferrin for iron [30]. In addition, mTOR activation increases angiogenesis via translation of VEGF, thus increasing the influx of nutrients used to generate ATP. On the other hand, when resources (ATP, oxygen, nutrients, etc.) are low, the function of mTOR is inhibited under the influence of the TSC complex, thus ensuring retardation of the biological processes [31]. It has been shown that both Akt and mTOR are linked to each other via positive and negative regulatory circuits, which restrain their simultaneous hyperactivation in normal cells. There is increasing evidence indicating that the activation of the mTOR pathway induces inhibitory signals to the PI3K/Akt pathway. This negative regulation occurs through IRS proteins, a family of adapter

proteins essential for mediating the effects of insulin signalling and PI3K pathway activation [3].

The VHL/HIF pathway

The importance of angiogenesis and its associated pathways cannot be overstated in the current era of targeted molecular therapeutic development. For example, the role of *VHL* gene and protein in the pathogenesis of clear-cell RCC has transformed the understanding of RCC tumour biology. VHL and its interactions with other molecules, such as HIF affect the vital molecular processes of angiogenesis, glucose metabolism, cell proliferation and apoptosis. *VHL* encodes a tumour-suppressor protein which in its native form typically forms multimeric complex with several other moieties (Elongin B, Elongin C, Cul2 and Rbx1) and binds to HIF-1α in the setting of hypoxia [32]. Normally, VHL directs HIF-1α towards degradation, while VHL alterations prevent the degradation of HIF-1α [33]. Increased binding of HIF-1α to hypoxia-response elements leads to the transcription of HIF target genes such as VEGF or carbonic anhydrase 9 (CA-9) [34].

HIF-1 has been studied as a prognostic marker, and patients with locally aggressive RCC and poor prognosis have lower levels of HIF-1α expression [35]. VEGF expression has been related to RCC aggressiveness [36], as well as overall prognosis in patients with advanced disease treated with anti-angiogenic therapy [37]. There are conflicting reports about the prognostic role of CA-9. Older immunohistochemical studies associated high CA-9 expression with increased survival in patients with clear-cell RCC and treated with immunotherapy [38,39]. However, a recent prospective study found that increased CA-9 expression was associated with a decreased overall response rate [40]. Other novel potential applications for CA-9 include the development of a CA-9 fusion antibody for functional imaging [41] and the use of a humanised monoclonal antibody to CA-9 given as an adjuvant targeted therapy after nephrectomy [42–44].

Cell-cycle markers

Increased *p53* staining has been linked to more rapid progression and reduced survival in RCC [45,46]. Increased expression of p21, a downstream target of p53 which inhibits cyclin-dependent kinases and plays a role in apoptosis, has been observed in RCC cell lines [47] and correlates with worse survival in those with metastatic RCC [48]. The loss of another cell-cycle inhibitor, p27 and the subsequent loss of cell-cycle regulation have been correlated with RCC recurrence [49]. Ki-67, a well-studied proliferation marker, was shown to be an independent predictor of disease-free survival in localised RCC [45,50], and higher Ki-67 expression was correlated with higher tumour grade and worse prognosis [51].

Apoptotic markers

The end-game of cellular self-management is the ability to dictate controlled cell death, or apoptosis. The pathways of apoptosis have been widely implicated in a variety of malignancies, and in RCC might have prognostic utility in risk assessment. Over-expression of Bcl-2, a negative regulator of apoptosis, has been reported in up to 70% of RCC specimens, and might promote tumorigenesis and explain the relative resistance of RCC to standard cytotoxic therapy [52]. Patients harbouring tumours with methylated APAF1, another apoptotic marker, had a greater risk of recurrence and disease-specific death from RCC [53,54]. Increased survivin expression, another inhibitor of apoptosis, has also been independently associated with higher stage and grade and lower disease-specific survival from RCC [55–58]. A recent study showed that mTOR activation of pS6K increases protein levels of survivin, which blocks extrinsic and intrinsic apoptotic pathways, showing the role of mTOR in cell survival [59].

Gene expression profiling

Recently gene expression profiling was used to predict RCC prognosis or molecular targets for therapy [60,61]. Another exciting new avenue of research into prognostic markers in RCC comes from a prospective study investigating individual RCC tumour genomes in nephrectomy specimens from the patients who were treated before surgery with the approved anti-angiogenic targeted therapies, sunitinib and everolimus. That study hoped to identify new biomarkers predicting drug response or resistance, and potentially discover new therapeutic targets in RCC [62]. Recent data from a similar study correlating germline polymorphisms to the outcomes in patients with RCC receiving pazopanib, an oral anti-angiogenic targeted therapeutic agent, identified a specific polymorphism in the *IL-8* gene which predicted disease progression, and another polymorphism in *HIF-1α* that predicted the response rate to therapy [63]. Similarly, there is an increasing interest in the utility of synthetic lethality screens for individual patient's tumours that can identify the most appropriate areas for therapy [13]. This is the next logical extension of biomarker utility, where physicians can synthesise all available patient information, their host response and the tumour's biology to provide personalised therapy for RCC.

Biomarker scores and integration into RCC prognostication

The ultimate goal of developing biomarkers is the clinical application. The best example of synthesising pathological, clinical and molecular markers into one

prognostic schema was from Kim et al. [64], who developed a nomogram predicting disease-specific mortality rates in metastatic RCC which takes into account T-stage, performance status, CA-9, vimentin, p53 and PTEN. This model outperformed the validated University of California Los Angeles Integrated Staging System [65,66], which only takes into account TNM stage, Fuhrman grade and performance status. Despite its limitations, the prognostic model of Kim et al. provides an excellent foundation from which future investigators can incorporate more markers to most accurately provide prognostic information. A panel of three biomarkers, B7-H1, survivin and Ki-67, has been developed and termed the BioScore scoring system [67]. The hope is to augment the currently existing risk-assessment tools; however, the BioScore still requires external validation. We recently applied immunohistochemistry for mTOR, Raptor, p-4E-BP1, PI3K and PTEN on tissue microarray constructs of 258 clear-cell RCCs from patients treated with radical or partial nephrectomy. The relationship between the prognostic marker score, based on the number of altered markers (favourable, < three altered biomarkers; unfavourable ⩾ three altered biomarkers) and oncological outcome, was assessed. The cumulative number of aberrantly expressed constituents of the mTOR pathway correlated with aggressive tumour biology and inferior oncological outcomes [68]. These preliminary data support a prospective pathway-based exploration of the mTOR signalling cascade to augment the current clinicopathological predictors of oncological outcomes in RCC.

Many of the biomarker studies in RCC are retrospective. We need to discover promising markers arising from these studies and apply them prospectively to move to the stage of clinical application. The utility of molecular markers will surely involve combining them with the known clinicopathological prognostics. Moreover, their potential use in predicting the response to targeted molecular therapeutics for RCC must be extensively studied to provide personalised cancer therapy integrating different choices of surgical and medical methods.

Conclusions

Increased knowledge about molecular alterations and genetic changes is essential for improving the clinical care of patients with RCC. We are in an exciting period of discovery of more molecular markers for RCC that might improve the prognosis and potentially predict the response to targeted therapies. The mTOR pathway is highly implicated in RCC tumorigenesis and progression, and its constituents might represent a promising prognostic tool for RCC. Combining these newly discovered molecular markers with classic clinicopathological variables might potentially improve the prognostication and management of patients with RCC.

Conflict of interest

None of the authors of this manuscript have any financial or personal relationships to disclose that could inappropriately influence or bias our work.

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