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Review

Resistance to tyrosine kinase inhibitors in clear cell renal cell carcinoma: From the patient's bed to molecular mechanisms☆

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ABSTRACT

The introduction of anti-angiogenic drugs especially tyrosine kinase inhibitors (TKIs) was a breakthrough in the treatment of renal cell carcinoma (RCC). Although TKIs have significantly improved outcome in patients with metastatic disease, the majority still develop resistance over time. Because different combinations and sequences of TKIs are tested in clinical trials, resistance patterns and mechanisms underlying this phenomenon should be thoroughly investigated. From a clinical point of view, resistance occurs either as a primary phenomenon (intrinsic) or as a secondary phenomenon related to various escape/evasive mechanisms that the tumor develops in response to vascular endothelial growth factor (VEGF) inhibition. Intrinsic resistance is less common, and related to the primary redundancy of available angiogenic signals from the tumor, causing unresponsiveness to VEGF-targeted therapies. Acquired resistance in tumors is associated with activation of an angiogenic switch which leads to either upregulation of the existing VEGF pathway or recruitment of alternative factors responsible for tumor revascularization. Multiple mechanisms can be involved in different tumor settings that contribute both to evasive and intrinsic resistance, and current endeavor aims to identify these processes and assess their importance in clinical settings and design of pharmacological strategies that lead to enduring anti-angiogenic therapies.

Contents

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1. Introduction — resistance as a clinical problem in renal cell carcinoma treatment

The findings about abnormal activities of signal transduction pathways in clear cell renal cell carcinoma (ccRCC) have allowed to establish novel targeted therapies for this disease, greatly improving the treatment options and prognosis of RCC patients [1–4]. Angiogenesis is necessary to support the growth of ccRCC greater than 1 to 2 mm in diameter and its high activity is mostly mediated by mutation or epigenetic inactivation of the von Hippel Lindau (VHL) tumor suppressor gene and subsequent up-regulation of hypoxia-inducible factor (HIF) expression [5]. Overexpression of HIF protein results in an increased expression of pro-angiogenic VEGF and platelet derived growth factor (PDGF) — key players involved in ccRCC development and progression [5,6].

Clinical studies over the last few years have demonstrated that multiple agents effectively blocking the angiogenic pathway have clinical efficacy; these agents include TKIs (sunitinib, sorafenib, pazopanib, and axitinib), the anti-VEGF monoclonal antibody — bevacizumab (administered with interferon α) and mammalian target of rampamycin (mTOR) inhibitors — everolimus and temsirolimus [7,8]. Each agent offers a significant clinical benefit, determined by the rate of objective responses (OR), reduction in tumor burden (RECIST), and extension of progression-free survival (PFS) compared with the standard of care. The first approved drug in the TKI family, sorafenib, investigated in the TARGET randomized, double-blind, phase III study in patients refractory to cytokine therapy, gave a significantly prolonged PFS in comparison with placebo (5.5 *vs.* 2.8 months, $p < 0.001$) [1]. Another TKI, sunitinib, compared with INF-α in a randomized phase III trial, has shown a significant prolongation of PFS (11 *vs.* 5 months, $p < 0.001$) in previously untreated patients with ccRCC [3]. This trial also demonstrated longer OS in the sunitinib group (26.4 *vs.* 21.8 months, $p = 0.051$), as well as significantly improved objective response rate (ORR) reaching 47% for sunitinib compared with 12% for INF-α ($p < 0.001$) [9]. Clinical outcomes for pazopanib treatment, notably prolongation of PFS in cytokine-pretreated patients compared to placebo (9.2 *vs.* 4.2 months, $p < 0.001$) and treatment-naïve population (11.1 *vs.* 2.8 months, $p < 0.001$), definitely confirmed the great potential of TKIs and raised hopes for a breakthrough in ccRCC treatment [4]. Based on the results from multiple clinical trials, current clinical practice guidelines recommend the use of VEGF inhibitors, sunitinib, bevacizumab with INF-α and recently pazopanib in first-line therapy for patients with metastatic RCC (mRCC) with good or intermediate prognosis according to MSKCC [10,11]. TKIs are also currently used as standard treatment for patients after previous cytokine therapy and alternatively in sequence (with change of the TKI) in second-line treatment [12].

Despite the therapeutic progress, complete and durable responses have been noted in only a few cases [10], necessitating chronic therapy for the majority of RCC patients, which is often associated with significant toxicity [1,3]. Another issue is that the response to treatment with a specific agent differs between patients, suggesting specific molecular mechanisms promoting individual susceptibility to each TKI. A large systematic review by Park et al. analyzed clinical data from over 12 clinical centers of patients with ccRCC treated with TKIs and showed that 26% out of 1056 patients treated with sorafenib and sunitinib were primarily refractory to treatment, showing no disease stabilization nor clinical benefits. The majority of these VEGF-refractory patients exhibited a uniform poor outcome regardless of therapy received [14]. While some mRCC patients are primarily refractory to VEGF-targeted treatment, the rest who primarily respond to VEGF-targeted treatment often develop secondary (acquired) resistance to certain agents after prolonged treatment. Typically ccRCC patients develop resistance to various TKIs within a median of 6–12 months [8], at which point tumor growth resumes despite continued administration of the drug, causing progressive disease [14].

Therefore, a pressing clinical and scientific question arises about the mechanisms determining resistance to TKI therapy, and the relevant treatment approach for metastatic ccRCC patients. To date, emerging clinical and preclinical data are available to address this issue. In this review, we analyze the existing data from both fields that provide insight into clinical ccRCC treatment limitations and needs, as well as recent advances in the identification of resistance determinants on a molecular level. Filling the gap in understanding TKI resistance development is necessary to propose possible strategies for continuous and efficient treatment of patients with ccRCC and improving their dramatic prognosis.

2. Molecular mechanisms of RCC resistance

In general, tumor sensitivity to targeted agents occurs when its growth and progression depend on the constitutive activity of signaling pathways specifically targeted by these agents. On the other hand, resistance may occur 1) when targeted proteins are inaccessible for drug binding due to their structural alteration or 2) upon activation of an alternative signaling pathway(s) or 3) due to upregulation of specific molecule expression that compensates for drug-mediated inhibition [13]. Based on the results of both preclinical and clinical studies indicating drug exposure-dependent origin of resistance occurring in RCC treatment [14–16], two general models of tumor resistance to anti-angiogenic agents targeting the VEGF pathway have been postulated: an adaptive (evasive) resistance, which occurs after a prolonged application of a drug (providing a period of tumor control), and intrinsic (preexisting) non-responsiveness despite the presence of an active agent, showing no therapeutic benefit [17].

2.1. Acquired resistance

The traditional concept of drug resistance being acquired by either mutations within genes encoding a drug target or by genetic alterations in mechanisms determining drug uptake and efflux has become less probable in the light of new preclinical and clinical data [18,19]. Although experimental evidence is not yet definitive, various studies suggest that at least four distinct mechanisms mediate acquired resistance to VEGF-targeted therapies; these are 1) up- or downregulation of genes involved in the alternative signaling pathway supporting angiogenesis in the tumor environment; 2) increased pericyte coverage of tumor vessels; 3) recruitment of pro-angiogenic inflammatory cells from bone-marrow; and finally 4) increased invasiveness of tumor cells into the normal tissue, which obviates the need for neovascularization [17]. There are studies that postulate that mechanisms involved in multi-drug resistance determining RCC resistance to chemotherapy might be also involved in decreased intake of TKIs (5) [20]. Finally the study of Gotink introduced the mechanism of lysosomal sequestration as a specific cellular adaptation to the toxic TKIs concentration in TKI-resistant renal cell cancer *in vitro* models [21]. This paragraph aims to describe the role of each mechanism mentioned above in acquisition of TKI-specific resistance and explain the rationale behind them.

2.1.1. Angiogenic switch — activation of alternative pathways supporting angiogenesis

A noticeable inhibition of tumor growth followed by its restoration after prolonged treatment is commonly seen by clinicians during TKI treatment in responding RCC patients. Preclinical studies investigating this phenomenon indicate that the angiogenesis switch may be determined by both overexpression of factors involved in alternative pro-angiogenic pathways and by downregulation of angiostatic ones [22].

The evidence for the evasive resistance being mediated by an alternative signaling pathway comes primarily from the preclinical study of Casanovas et al. performed on the genetically engineered *Rip1–Tag2* mouse model of pancreatic neuroendocrine (islet cell) cancer

[23]. They performed DC101 antibody-mediated blockage of the VEGF signaling pathway (VEGFR2 in particular) generating a predictable transient (10–14 days) attenuation of tumor growth and decrease in tumor vascularity. During the following tumor regrowth and restoration of tumor vasculature, mRNA analysis revealed a significant overexpression of pro-angiogenic factors, notably fibroblast growth factor 1 and 2 (FGF1/2), ephrin A1 and A2 (Efna1/2) and angiopoietin 1 (Ang1) compared with untreated tumors. Upregulation of these genes may be correlated with episodes of acute hypoxia evoked by anti-angiogenic treatment. Interestingly, simultaneous treatment with both VEGFR inhibitor and FGF suppressor (FGF-Fc fusion protein) caused a noticeable attenuation of the revascularization and hampered tumor growth indicating a contribution of FGF signaling to angiogenesis restoration. These findings are consistent with a clinical study of glioblastoma patients treated with another VEGFR inhibitor — cediranib, where blood analysis showed much higher levels of FGF in patients undergoing treatment relapse and progression compared to the same patients in the response phase [24].

Another factor that has been shown to play an important role in the maintenance of tumor angiogenic capability is the pro-angiogenic cytokine, interleukin 8 (IL-8). Mizukami et al. have shown that the use of neutralizing anti-IL-8 antibody blocked tumor angiogenesis in colon cancer cell lines with HIF-1α knockdown preserving VEGF expression [25]. Further corroboration comes from the study on another tumor model, that implicated the link between sunitinib resistance and increased level of IL-8 in plasma, showing that antibody-mediated neutralization of IL-8 causes tumor re-sensitization to sunitinib treatment [16]. This direct correlation between IL-8 plasma level and sunitinib resistance supports the concept of that IL-8 levels may be a predicting factor for clinical response to sunitinib and may possibly help to identify either reverse acquired or intrinsic resistance to sunitinib in RCC patients [26].

Hypoxia seems to be a driving force for activation of alternative pathways and proteins supporting tumor angiogenesis. It has been found to induce placental growth factor (PlGF), most probably involved in potential alternative signaling in VEGF therapy-resistant tumors. Notably, neutralization of PlGF has been shown to prevent macrophage infiltration towards severely hypoxic tumors, thus obviating an angiogenic rescue program determining resistance to VEGFR inhibitors [27]. A study in a mouse model showed that VEGFR1 and VEGFR2 transphosphorylation was achieved *via* PlGF and that treatment with recombinant PlGF stimulated revascularization of hypoxic tissues [28,29]. This is consistent with the reports of both glioblastoma and mRCC patients who showed increased levels of PlGF and VEGF in the blood upon bevacizumab or other receptor TKI treatment [24,27,30]. These data provide a starting point to conceptualize that PlGF-targeted therapy combined with existing anti-VEGF strategies could be promising in overcoming TKI resistance in RCC malignancy.

Another system that acts synergistically with VEGF to stimulate tumor angiogenesis by controlling endothelial cell survival and vascular maturation is angiopoietin/Tie signaling. This pathway involves tyrosine kinase receptors Tie1 and Tie2 with corresponding Ang-1 and Ang-2 specific ligands. Plasma levels of Ang-2 were found to be decreased in patients with mRCC during the responsive stage of sunitinib therapy, while during development of sunitinib resistance, a significant increase in Ang-2 plasma concentration was observed [31]. This suggests a role for Ang-2 in adaption to VEGF-blockage, which *via* its binding to Tie2 promotes vessel assembly through a direct simulation signal to endothelial cells and initiation of the angiogenic switch [31,32]. Ang-2/Tie2 signaling may therefore have an angiogenic potential that can parallel the VEGF-dependent pathway. Attenuation of Ang-2 by a new class of biotherapeutics called CovX-Bodies (protein-antibody construct) showed a significant inhibition of tumor vessel density and when combined with sunitinib and sorafenib it provided even greater tumor shrinkage [33]. A similar correlation with resistance development was also observed in terms of sphingosine kinase associated with cell proliferation, survival and angiogenesis [34]. Bhatt et al. have identified a clear correlation between increasing levels of plasma sphingosine 1-phosphate — S1P (product of the aforementioned enzyme) and sunitinib resistance development [35].

Further evidence of the transient nature of the angiogenesis escape mechanisms has been provided by Bender and Ullrich, who demonstrated a correlation between upregulation of *PRKX*, *TTBK2* and *RSK4* gene expression and sunitinib resistance [36]. PRKX (protein kinase, X-linked) apart from being involved in renal development, regulation of epithelial cell migration and induction of glomeruli formation, also manifests its oncogenic potential by activation of MIFT (microphthalmia-associated transcription factor); its knock-down is sufficient to repress tumorigenesis *in vivo* [37]. To date mutations and/or aberrations in several members of the MIFT gene family have been reported in papillary renal cell carcinoma (TIE3, TFEB) and melanoma (MIFT), which further supports their role in oncogenesis [38–40]. Furthermore Bender and Ullrich's study proved the crucial role of PRKX in development of sunitinib resistance in a *PRKX* knock-down experiment, which was sufficient to resensitize renal cell carcinoma cells. A similar, but slightly lower sensitization effect was noticed in a knock-down experiment on *TTBK2*, whose product is involved in similar regulation pathways as PRKX. Interestingly, *TTBK2* expression pattern during sunitinib resistance appeared to be different from the one observed in cell lines showing sorafenib resistance, suggesting divergent mechanisms of sunitinib and sorafenib resistance development [36]. The *RSK4* gene also seems to be associated with oncogenic transformation since its aberrant expression has already been identified in several cancer models, e.g. breast cancer [41]. Moreover, other members of this RSK family, notably RSK1 and RSK2, have been shown to activate Creb and MIFT factors involved in the Ras/Mapk signaling pathway [42–44]. Finally, a complex blockage of *PRKX*, *TTBK2* and *RSK4* apart from sensitization to sunitinib resulted in improved anti-migratory effect of sunitinib in renal cancer and melanoma cells [36]. These data clearly suggest that PRKX, TTBK2 and RSK4 are involved in one complex signaling mechanism that not only transiently affect sunitinib-mediated apoptosis but also functions in mediating a cancer type independent overall sunitinib insensitivity. Therefore this set of genes might represent targets for development of novel treatment strategies supporting sunitinib anti-angiogenic potential and simultaneously overcoming resistance.

Angiogenesis as one of the survival-determining processes acquires its oncogenic potential not only when stimulated by alternative pro-angiogenic signaling but also when negative (angiostatic) regulation fails. Several studies performed on VEGFR2-TKI resistant HUVEC clones revealed a significant downregulation of the angiogenesis-associated genes, endothelial cell-specific molecule 1 (*ESM1*), homeobox A9 (*HOXA9*) and a platelet endothelial cell adhesion molecule 1 (*PECAM 1/CD31*) [45–47]. These findings are consistent with another work demonstrating a noticeable decrease in ESM1 plasma levels in patients with mRCC undergoing prolonged TKI treatment and showing symptoms of resistance [48]. ESM1 therefore might appear a promising biomarker for anti-angiogenic inhibitors. In turn, downregulation of the angiogenic homeobox gene *HOXA9* through EPHB4 was found to be responsible for downregulation of VEGFR2 in resistant clones. This specific quenching of VEGFR2 — the main "gate" for the conventional angiogenic signal was backed up by upregulation of *CXCL5*, *CXCL6* and *CXCL7* pro-angiogenic genes suggesting contribution of the inflammatory arm in resistance development [45,49].

2.1.2. Increased pericyte coverage of tumor vessels

A growing body of evidence supports the concept that some tumors rely on pericytes to maintain a core of stable and functional existing blood vessels during anti-VEGF therapy. This is coherent with clinical observation of several groups pointing a specific distinguishable morphology of surviving thin tumor vessels covered tightly with pericytes [17,50–52]. Pericyte recruitment appears to be a critical

support for vasculature in the absence of VEGF-mediated signaling, which is supported by the fact that vessels lacking pericyte coverage are more vulnerable to anti-VEGF therapy [17,53] and that pericytes are able to express considerable levels of VEGF and other factors supporting survival of endothelial cells [54,55]. However, in the case of renal cancer, active drugs — sunitinib and sorafenib target both VEGFR and PDGFR receptors, attenuating PDGF-mediated pericyte induction. On the other hand, TKI-dependent pericyte development blockage may cause a severe reduction of these cells and consequently disrupt the vascular integrity, promoting tumor cell transition into the blood circulation, thereby facilitating metastasis [17]. In the study of Xian et al. on the *Rip1–Tag2* pancreatic islet mouse tumor model genetic disruptions of pericyte coverage were associated with increased metastasis [56].

2.1.3. Recruitment of pro-angiogenic inflammatory cells from bone-marrow

Hypoxia caused by the regression of tumor vasculature during the course of anti-angiogenic treatment leads not only to enhanced pro-angiogenic factor production within the tumor, but also to the recruitment of different bone marrow-derived cells (BMDCs). These BMDCs involve pro-angiogenic tumor-associated macrophages [57], $VEGFR1^+$ hemangiocytes [58,59], immature monocytic cells (so-called TIE^+ monocytes) [60] and $CD11b^+$ myeloid derived suppressor cells (MDSCs) [61,62]. They primarily act as vascular modulators responsible for the expression of a variety of cytokines, growth factors and proteases supporting vasculature remodeling [63,64]. Given the immunosuppressive and pro-angiogenic features of MDSCs, it is reasonable to suggest that they might be involved in resistance development in patients treated with sunitinib [65,66]. The concept of hypoxia-dependent BMDC recruitment comes from observations of an *in vitro* ischemic model, where the recruitment of endothelial progenitors and CXCR4 + BMDCs was found associated with increased levels of HIF-1α and SDF1α (a ligand to CXCR4) [67,68]. In the study of glioblastoma multiforme (GBM) characterized by extensive hypoxia, HIF-1α was found to induce recruitment of pro-angiogenic bone marrow-derived $CD45^+$ myeloid cells including those expressing TIE2, VEGFR1 and CD11b together with $F4/80^+$ tumor-associated macrophages [63,69]. In terms of renal cancer also associated with high HIF-1α levels, Farace et al. have noted that higher expression of $CD45^{dim}CD34^+VEGFR2^+$ circulating progenitor cells in peripheral blood was correlated with poor PFS and OS in patients with mRCC treated with sunitinib [70]. The correlation between therapy-induced hypoxia and the recruitment of BMDC has been supported by the fact that anti-angiogenic therapy-associated disruption in vasculature determines tumor acute secondary hypoxia conditions, which are triggered by further accumulation of BMDC factors involved in neovascularization [71]. Notably, treatment-naïve transplanted tumors have lower levels of BMDCs, which indicates that the recruitment of pro-angiogenic inflammatory cells from bone marrow is a tumor adaptive response to anti-angiogenic therapy.

2.1.4. Increased invasiveness of tumor cells into a normal tissue

In addition to the above-mentioned mechanisms for escape or evasion, there are increasing considerations about another insidious way of tumor adaptation to anti-angiogenic therapy occurring *via* an increased invasiveness. This was first noted in a study where attenuation of angiogenic factors such as VEGF, HIF-1α and matrix metalloproteinase 9 (MMP9) resulted in increased invasiveness and continued slow growth of tumors in the mouse model of GBM [72]. This invasiveness of glioblastoma cells was reflected in their co-opting of normal blood vessels and using them for a deeper brain invasion achieving in this case a dispersed phenotype [63,73]. In turn, Hammers et al. in their study have reported that the onset of the epithelial–mesenchymal transition (EMT) phenotype in patients with ccRCC with sunitinib resistance was associated with reversion of previously acquired resistance. Establishment of human tumor xenografts from mRCC with acquired resistance to sunitinib has shown reversion to an epithelial histology with sensitivity to this drug when implanted into a new mouse microenvironment [74]. Sunitinib-resistant clones were found to downregulate platelet endothelial cell adhesion molecule 1 (*PECAM1*)/*CD31*characteristic for the mesenchymal transition process [45]. A concurrent study of the sunitinib-resistant renal (ACHN) and prostate (DU145) model with experimentally-induced EMT (by overexpression of *Twist*, *Snail* and *ZEB1*) xenografted into NOD/SCID mice has shown that ACHN-Twist and DU145-ZEB1 were able to grow upon continuous sunitinib treatment, while other tumors failed [75]. These data suggest that induction of EMT-associated genes by tumors may simultaneously activate signaling responsible for resistance regulation. Reversal of the histological phenotype identified in mouse xenografts indicates that the escape mechanisms to TKI therapy are of a transient nature. However, the clinical significance of this discovery has to be carefully assessed, considering that the systemic nature of this phenomenon may differ between animal models and human cases.

2.1.5. Chemotherapy resistance vs. TKI-resistance

The "kidney" nature of RCC is characterized by a well-developed secretory apparatus with high expression of membrane proteins specialized for elimination of xenobiotics. This "attribute" appears to determine the high, unmanageable kidney cancer resistance to chemotherapeutic agents [20]. Thus, mechanisms determining such an effective system for chemotherapeutic agent elimination should be addressed to identify all possible determinants of TKI resistance. Several studies imply that TKIs are recognized by the cellular mechanisms handling xenobiotics, which address a question about the actual TKI pharmacokinetics within the tumor. These membrane structures conferring multidrug resistance in cancers are ATP-binding cassette (ABC) drug transporters with the most prevalent — P-glycoprotein (P-gp, ABCB1), multidrug resistance associated protein (MRP) 1 (ABCC1) and ABCG2 (breast cancer resistance protein, MXR) [76]. There is a growing body of evidence that they are important determinants of absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) of several TKIs such as gefitinib, EKI-485, erlotinib, imatinib, nilotinib, CI1033, INNO-406 [77–82]. Sunitinib at nontoxic concentrations (2 μM) was found to block P-gp- and ABCG2-mediated efflux of xenobiotics. This suggests a direct interaction between sunitinib and P-gp and ABCG2, respectively, and induction of conformational changes similar to classic substrate/inhibitor blockage. Sunitinib-mediated inhibition results in a partial reversion of the P-gp-mediated resistance and complete reversion of the ABCG2-mediated one, with no effect on ABCB1-, ABCC1- and LRP-mediated multidrug resistance [82,83]. Further investigations have shown that a specific germ-line mutation of the *ABCG2* gene (1291T>C) causing a single amino acid substitution of F431L can abolish the inhibitory effect of sunitinib on ABCG2 activity [84]. These examples might be considered as side effects of sunitinib application surprisingly helping to overcome chemo-resistance, but at the same time trapping an active drug compound within various MDR structures diminishing its main anti-angiogenic potential. Another study has identified sunitinib and sorafenib as substrates for an efflux transport mediated by RLIP76 — a stress-responsive membrane protein [85]. Considering the high prevalence of RLIP76 in kidney cancer cells compared to normal tissue and the tumor repression effect observed upon specific RLIP76 inhibition/depletion *in vivo* experiments, it seems reasonable that the cyto-protective role of RLIP76 might play an important role in renal cancer.

2.1.6. Lysosomal sequestration of sunitinib

An intriguing report by Gotink et al. identified a novel mechanism of sunitinib-mediated resistance based on lysosomal sequestration. This preclinical study showed that in patients treated with sunitinib the intratumoral concentration of this drug tends to be 10-times higher

compared to the plasma level. Fluorescent imaging of sunitinib intracellular fate in cells which eventually developed resistance to this drug has revealed a clear sunitinib distribution to acidic lysosomes characteristic preferably for the resistant clones. The hydrophobic nature of sunitinib enables this molecule to easily cross the lysosomal plasma membrane, but protonation in the acidic compartment prevents it from leaving, supporting the thesis of sunitinib sequestration. Such a mechanism protects cells from anti-angiogenic activity of sunitinib despite its high intracellular concentration providing a new model of transient acquired resistance [21].

2.2. Intrinsic resistance

The mechanisms of intrinsic resistance seem to be even more mysterious than those determining the evasive one. The observations of patients' unconditional non-responsiveness to VEGF-targeted therapy indicate rather the permanent nature of this condition more likely determined by individual molecular features of each patient. Moreover, the primary resistance in some patients could be relative. It is possible that the VEGF pathway in patients treated with TKIs may remain activated due to inadequate and/or insufficient suppression of the particular drug. These variations in patient response might be determined by the individual pharmacokinetic variability and/or VEGF receptor polymorphism or by an immunological background and/or unbalanced regulation of apoptosis [32].

2.2.1. Immunomodulatory effect

Intrinsic resistance may be simply related to the existing plethora of immunogenic agents involved in control of angiogenesis either upstream or downstream from the inhibited agent. For example, a significant impediment to RCC immunotherapy has been associated with the role of myeloid — origin cells being potent suppressors of tumor immunity. Myeloid derived tumor cells (MDSCs) present in the blood, lymph nodes and bone marrow of various cancer patients have been shown to inhibit both NK and $CD4^+$ and $CD8^+$ T cells and macrophages with simultaneous stimulation of regulatory T cells [86]. A preclinical study of Shojaei et al. showed that a subset of murine transplanted tumors grown in mice is non-responsive to an anti-mouse VEGF antibody treatment. This intrinsic resistance was found to be due to a CD11b + Gr + population of MSDCs that infiltrated the tumor during the course of previous chemotherapy treatment. This CD11 + Gr + subpopulation was shown to express a number of pro-angiogenic factors, giving pretreated tumors an advantage over non-pretreated ones in terms of survival upon subsequent chemotherapy [87]. Several tumor-derived products (including VEGF) block myeloid cell differentiation and facilitate accumulation of a heterogeneous group of immature myeloid cells within the tumor [88–91]. The CD11 + GR1 + population of MDSCs in particular have a capacity to increase intratumor vascular density, reduce necrosis, and noticeably stimulate tumor growth [62,92]. The advent of TKIs has shown that MDSCs can be targeted by sunitinib via their VEGFR receptors and sunitinib treatment was shown to be significantly reduced MDSCs accumulation in peripheral blood and reversed T cell (INF-γ) suppression in both mRCC patients and murine tumor models [65,93]. Finke et al. have also shown that sunitinib was ineffective at reducing MDSCs ($Gr1^+CD11^+$ population) only in 4T1-bearing mice. This tumor-specific persistence of MDSCs was associated with decreased intratumor T cell INF-γ response and more importantly with an increased granulocyte macrophage–colony stimulating factor (GM-CSF) expression [65]. Another subset of cells hampering immune anti-tumor response is CD4 + regulatory T cells highly prevalent in tumor peripheral blood, directly reducing the level of conventional T cells (by expression of I-10, TGF-β and CTLA4). As mentioned before, T reg high expression is stimulated by MDSCs. On the other hand, the subset of myeloid dendritic cells (mDCs) which enhance both innate and acquired immunity against tumors were found to be inhibited by VEGF [94]. Another tumor-specific interaction that has been considered for immune system suppression (inhibition of T cells) is mediated by interaction between programmed death ligand 1 (PD-L1) and programmed death 1 (PD-1) receptor present on T cells, B cells, monocytes and endothelial cells. Therefore, blockage of PD-1 receptor on activated T cells through BMS-936558 and CT-011(pidilizumab) human anti-PD-1 monoclonal antibodies (mAbs) has been proposed as a potential immunotherapy for patients with solid tumors including RCC [95,96]. Blockage of PD-1 function results in attenuation of apoptotic processes in lymphocytes, primarily effector/memory T cells as well as augmentation of anti-tumor activities of NK cells. As a result, enhanced activities of T and NK cells lead to the intensification of anti-RCC immune response and the generation of tumor-specific memory cells.

RCC-specific immunogenic properties are also related to the specific set of HLA molecules expressed on its surface. Several immuno-modulatory molecules have been identified on the surface of renal tumor cells, including HLA-G and HLA-E previously thought to be specifically expressed only on extravillous trophoblast. HLA-G/E-mediated immune tolerance is normally based on a counter-attack of maternal immune surveillance, that enables an early tolerance and later acceptance of the semiallogenic fetal graft. However, these mechanisms seem to be hijacked by tumor cells to thwart immune response [97,98]. These examples clearly indicate a tumor-mediated switch of the immune system to a “tumor-protective” type, which supports tumor angiogenesis independently of the TKI treatment applied.

2.2.2. Apoptosis

In the majority of cancers, but especially in RCC, unbalanced regulation of apoptosis contributes to cancer growth by abnormally extending cell viability and facilitating higher rate of survival even upon cancer therapy. Some members of the tumor necrosis factor (TNF) family and TNF-related apoptosis-inducing ligand (TRAIL) proteins involved in apoptotic signaling are molecular markers being targeted in some malignancies; however their possible role in resistance of RCC to therapies remains controversial [99]. For example, TNF was found to be involved in induction of suppressor cells that inhibit tumor growth in myeloid tumors, in others being responsible for promoting tumor growth via activation of inflammatory response. Notably, it hampered cell-mediated immunity (squamous cell carcinoma) or was found to induce metastases by stimulation of S100A8/A9 triggering subsequent recruitment of tumor cells (lung cancer). Additionally it has been shown that application of TNF inhibitors results in cancer stabilization (ovarian cancer) [100]. TRAIL-based therapeutics, including recombinant TRAIL, TRAIL-receptor agonistic antibodies and TRAIL gene therapy are now being evaluated in clinical trials [101]. Since TRAIL-targeted therapy is often associated with noticeable resistance to TRAIL inhibitors, Yang and colleagues proposed the use of acrolein (a toxic product of lipid peroxidation) to sensitize renal cancer Caki cells to TRAIL-induced apoptosis [102].

Other mechanisms that determine cancer development and may be involved in resistance development include altered cell cycle checkpoint genes, faulty DNA repair genes and drug efflux pumps and increased or altered drug targets. A new drug bortezomib inhibits the ubiquitin–proteosome system preventing the degradation of regulatory cell cycle control proteins, as well as interfering with gene expression, cell adhesion molecules, metastasis and angiogenesis. Brooks et al. have shown bortezomib-dependent sensitization of RCC to TRAIL apoptosis through increased activation of caspase-8. It has also been noted that bortezomib activity was only restricted to the extrinsic apoptotic pathway utilizing cell surface receptors, whereas it did not elicit any changes in intracellular levels of B-cell lymphoma-2 (Bcl-2) and inhibitors of apoptosis (IAP) protein family members involved in intrinsic pathways [103]. On the other hand, high levels of Bcl-2 and/or Bcl-XL preventing apoptosis have been noted in RCC tumor samples

derived from patients with highly aggressive tumor phenotype indicating that intrinsic apoptotic pathways may play a role in progression and resistance to therapy of this tumor [104]. Other studies also highlighted the role of caspase-3 inactivation in induction of apoptosis, which may explain in part the resistance of RCC to cancer therapies and possibly determine potential targets in apoptosis signaling for overcoming resistance to RCC treatment.

Another factor mediating therapy-dependent apoptosis is the CD95 receptor responsible for activation of caspase signaling. *In vitro* studies have shown an upregulation of CD95-ligand and/or receptor in a range of drug-sensitive cancers (such as leukemias, hepatocellular carcinomas and neuroblastomas) undergoing cancer treatment and significantly lower levels of CD95 complex components in drug-resistant tumors [105]. Nevertheless, anticancer drug-induced upregulation of CD95 receptor and ligand sometimes appears to be insufficient for an effective activation of CD95-mediated apoptosis. This might be due to mutations in p53 (characteristic for cancers), which directly transactivates CD95 receptor [106]. Considering that p53 mutations are relatively rare in RCC, it seems that the signaling of a wild-type p53 becomes activated in response to cellular stress but fails to elicit an apoptotic transcriptional program [107]. Cash et al. described another mechanism of apoptotic resistance mediated by a mutation in the *Birt–Hogg–Dube* (*BHD*) gene associated with the BDH syndrome, a rare inherited cancer susceptibility disease promoting RCC in approximately 1/3 of cases. *BHD*-deficient cells exhibited defects in cell-intrinsic apoptosis and reduced expression of the BH3-only protein Bim. A reduced Bim level in turn was found associated with a loss of TGFβ-mediated transcription and chromatin modification [108]. These findings suggest several targeted therapeutics like BH3-only mimetics, autophagy inhibitors, and HDAC inhibitors in treatment of BHD-related renal cancers.

Current knowledge indicates that mechanisms mediating apoptosis may overlap these that determine development and persistence of resistance to chemotherapy in RCC; however, no study has revealed their relevance in the case of resistance to TKIs. Moreover, high RCC heterogeneity may cause a serious problem in identification of a reliable and uniform therapeutic target within the frequently mutated apoptotic pathway.

3. Summary

The presented data describing mechanisms involved in either intrinsic or acquired non-responsiveness to TKI treatment clearly show that the resistance to TKIs is a multifactorial and complex mechanism that involves different types of cells prevalent at the tumor site as well as recruited from outside the tumor microenvironment. RCC acquired resistance to anti-angiogenic treatment triggering mechanisms responsible for its survival in the presence of active drugs is reversible, as multiple studies have shown restoration of the specific TKI sensitivity after prolonged tumor deprivation of that agent. Considering TKI acquired resistance as a secondary effect of treatment, to date clinical investigations have only identified cases of RCC patients showing resistance to either sunitinib and sorafenib. Further molecular analysis of TKI resistance in RCC revealed its drug-specific nature. This indicates that different treatment strategies should be undertaken to target either sunitinib or sorafenib resistance, as each of these clinical conditions is mediated by a different signaling pattern. The summary of signaling involved in acquired resistance both to sunitinib and sorafenib is outlined in Fig. 1.

It is important to note that the figure does not necessarily represent the signaling in a particular TKI-resistance condition, but serves as a model of the summary of all known interactions. However, only identification of all possible signaling pathways (signaling molecules and receptors) that contribute to tumor evasion from TKI treatment can lead to an effective RCC treatment. On the other hand, the nature of the intrinsic resistance in RCC does not create many opportunities for possible treatment. The problem of the individuals' inherited mutations or genetic polymorphisms in TKI targets or enzymes responsible for TKI pharmacokinetics still remains elusive for current treatment of RCC. Since permanent intrinsic resistance is determined by individual genetic patterns, introduction of genetic screening of patients into the standard clinical care would help to identify each patient's susceptibility for TKI treatment before its application. It is crucial to note that although these two types of resistance (acquired and intrinsic) are mediated by different mechanisms, their effects may add the total response rate to TKI treatment in the individual patient's case. Proposed supplementary RCC treatment options are presented in Fig. 2.

4. Future directions

Treatment of RCC has made great strides in recent years. Targeted therapies hit the weak point of RCC — angiogenesis contributing to overcome the RCC treatment impasse. However, its long-term efficiency still leaves a great deal to be desired. The major issue in RCC treatment is still drug-specific resistance and current efforts aiming to overcome this problem are usually hampered by the excessive toxicities. Thorough consideration of the most adequate treatment strategy for RCC patients requires addressing some emerging questions: whether current TKI inhibition of selected agents in sufficient/efficient, whether the target is the same (equally available) in every patient and finally whether the target is an endothelial cell-, stromal cell- or tumor specific in certain tumors and metastases. Thus there is a pressing need for new clinical tools to assess the level of the patients' response to certain targeting agents and whether an alternative target would provide a greater clinical benefit.

The elusive holy grail of oncology clinicians would certainly be an indication for these patients, who have the highest chance of clinical advantage and/or the lowest chance of toxicity prior to treatment. Due to its permanent nature intrinsic resistance appears to be a troublesome issue in oncology clinical practice hampering effectiveness of even the most potent anti-angiogenic factors used in RCC treatment. A recent study of Gerlinger et al. also emphasizes the importance of examination of intratumor heterogeneity in tumor biopsies, as certain mutations in mTOR kinase and *SETD2*, *PTEN*, and *KDM5C* tumor suppressor genes show various prevalence even within the same tumor mass [109]. Making efforts to overcome primary non-responsiveness, the major focus should be put on evaluation of drug pharmacokinetics and target (VERF/R) polymorphisms in order to establish relevant biomarkers.

Considering the existing data, the most accurate strategy to overcome anti-VEGF escape mechanisms determining “evasive resistance” might be achieved by development of synergistic inhibition of the VEGF pathway [110]. Several escape mechanisms, recruitment of BMDCs, induction of pericyte coverage as well as an increased invasiveness correlated with ETM, are mostly triggered by acute hypoxia conditions caused by anti-VEGF therapy. Thus, inhibition of tumor cell adaptation to therapy-induced hypoxia might be tackled by synergistic use of histone deacetylase (HDAC) and mTOR inhibitors blocking the HIF pathway [110]. Several preclinical trials have demonstrated an antitumor activity of HDAC inhibitors, notably SNDX 275 and vorinostat in TKI-refractory patients at various stages of RCC [111–113]. Sunitinib anti-VEGFR inhibitory effect could also be strengthened by addition of an antibody directly targeting VEGFR2 (DC101) in later phase of treatment in order to prevent the pericyte coverage effect mediating resistance [114].

Since a common feature for resistance development is an increased level of VEGF in plasma of RCC patients, the most rational approach seems to be a “sequential monotherapy approach” based on treatment with an antibody that neutralizes elevated levels of VEGF [32]. When the evidence suggests the acquisition of non-responsiveness to anti-TKI treatment mediated by activation of an alternative signaling pathway the use of a combinational approach would be the most relevant. This would provide more complex angiogenesis inhibition achieved by simultaneous use of HIF inhibitor or anti-VEGF ligand

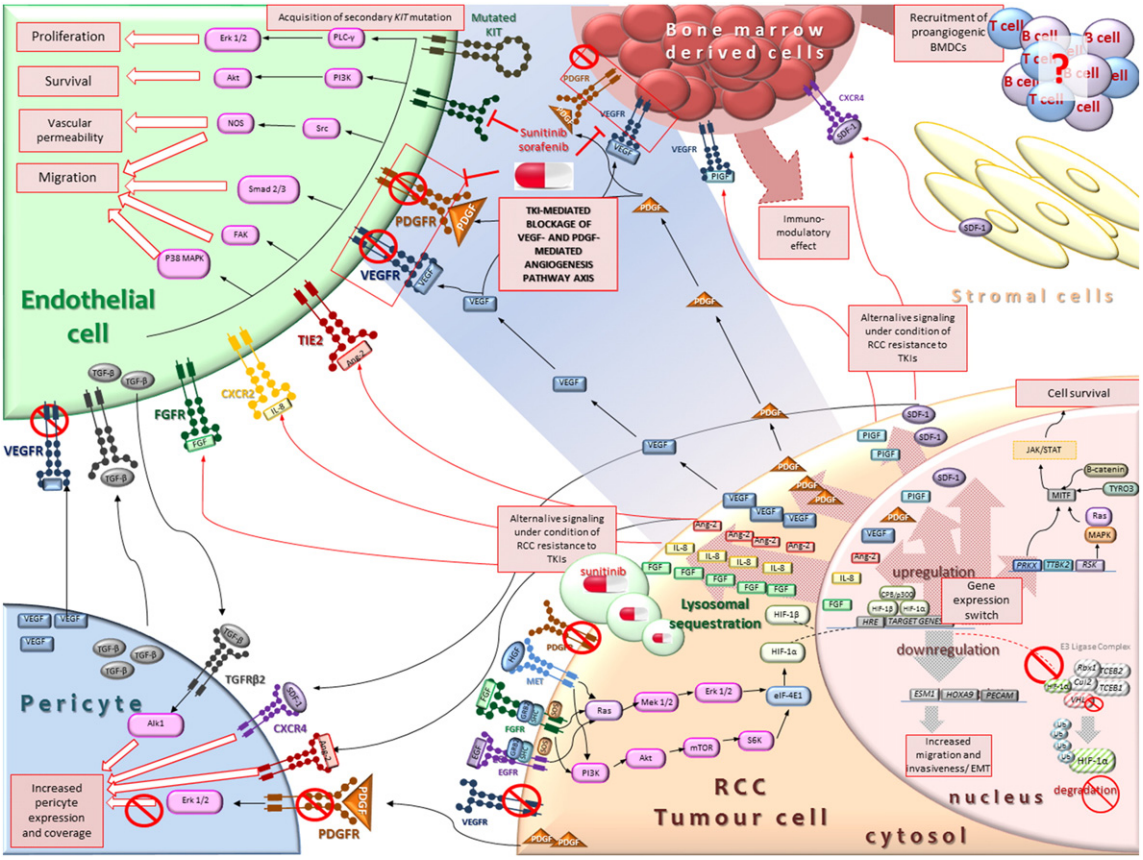

Fig. 1. Molecular pathways in TKI-resistant RCC. Abbreviations: Ang-2 — angiopoietin 2; BMDCs — bone marrow derived cells; EGF(R) — epidermal growth factor (receptor); EMT — epithelial mesenchymal transition; Erk 1/2 — extracellular-signal-regulated kinases 1/2; FAK — focal adhesion kinase-1; FGF(R) — fibroblast growth factor (receptor); HGF — hepatocyte growth factor; HIF-1α/β — hypoxia-inducible factor 1α/β; IL-8 — interleukin 8; MAPK — mitogen-activated protein kinase; MITF — microphthalmia-associated transcription factor; NOS — nitrous oxide systems; PDGF(R) — platelet derived growth factor (receptor); PI3K — phosphoinositide 3-kinase; PLC-γ — phospholipase C γ; PlGF — placental growth factor; RSK — ribosomal S6 kinase; SDF-1 — stromal derived factor 1; Smad 2/3 — small mothers against decapentaplegic kinase 2/3; TGF-β — tumor growth factor-β; TKIs — tyrosine kinase inhibitors; Ub — ubiquitin; VEGF(R) — vascular endothelial growth factor (receptor); VHL — von-Hippel Lindau factor.

antibody with the standard TKI, which might contribute to delay the occurrence of "evasive" resistance determined by the increased HIF dependency. This approach may also include a combination targeting alternative growth factors, such as FGF, PlGF, Ang-2 or IL-8. For example, a randomized, double-blind, placebo-controlled, phase II study evaluating efficiency of AMG 386 (sequestering Ang-1 and Ang-2) in combination with sorafenib has shown no improvements in PFS compared with sorafenib plus placebo; however, the observed increased ORR and reduction of tumor burden suggest an antitumor effect of AMG 386 in mRCC [115].

However, the biggest hurdle in developing combinational strategies is still the significant toxicity. Therefore in terms of increasing the patients' convenience during anti-angiogenic treatment, it seems the most rational to apply a sequential treatment strategy based on sequential switching to a drug with a different mechanism of action during the time of disease progression [116]. There is a strong body of evidence supporting this idea. For example, since sunitinib-specific inhibition of angiogenesis does not involve blockage of parallel regulatory pathways such as ERK-MAPK and Akt/mTOR, the use of the aforementioned pathway inhibitors might provide a wiser complementary model of therapy in TKI-refractory malignancies [117,118]. Another problem is the choice of the complementary/alternative agent. Results from an open-label, randomized, phase II study (TORAVA) evaluating combined therapies including temsirolimus and bevacizumab showed high toxicities for that regimen when compared with sunitinib, or bevacizumab with INF-α [119]. Similarly AEs were observed in a clinical trial testing a combination of sunitinib with bevacizumab [120], indicating that none of these strategies are effective in treatment of metastatic RCC patients. The studies of the cross-resistance between anti-angiogenic agents in resistant tumor cells have revealed that sunitinib resistant cell lines were cross-resistant to pazopanib and erlotinib, while remaining fully sensitive to sorafenib and everolimus [121]. The range of cross-resistance might be dependent on the specificity of these drugs. Therefore searching for new TKIs with varying specificities might bring a big promise to RCC refractory patients. One of those currently being tested — dovitinib, a new TKI, targets an additional receptor — FGFR (compared with "older TKIs"), whose mediated signal has been reported to be an important escape mechanism of anti-VEGFR therapies [122]. Application of everolimus and axitinib sequentially was found associated with a noticeable inhibition of PI3k/AKT/mTOR signaling [123,124]. This indicates the need to search for an individual strategy overcoming resistance to each TKI separately, since apparently different mechanisms mediate resistance to each agent.

A striking notion derived from analysis of existing data referring to the resistance to anti-angiogenic therapies is that the studies usually identify aberrant expression of single agents not necessarily involved in particular signaling and having known angiogenic related functions. Such various data give a scattered view of the possible mechanisms determining resistance development and regulation. In this light, it appears crucial to combine findings referring to abnormal gene expression (with relevant quantification and potential significance of each event) with data referring to abnormal levels of effector proteins for each cancer type. This would not only help to fill the gaps in our understanding of resistance but possibly also determine new signaling

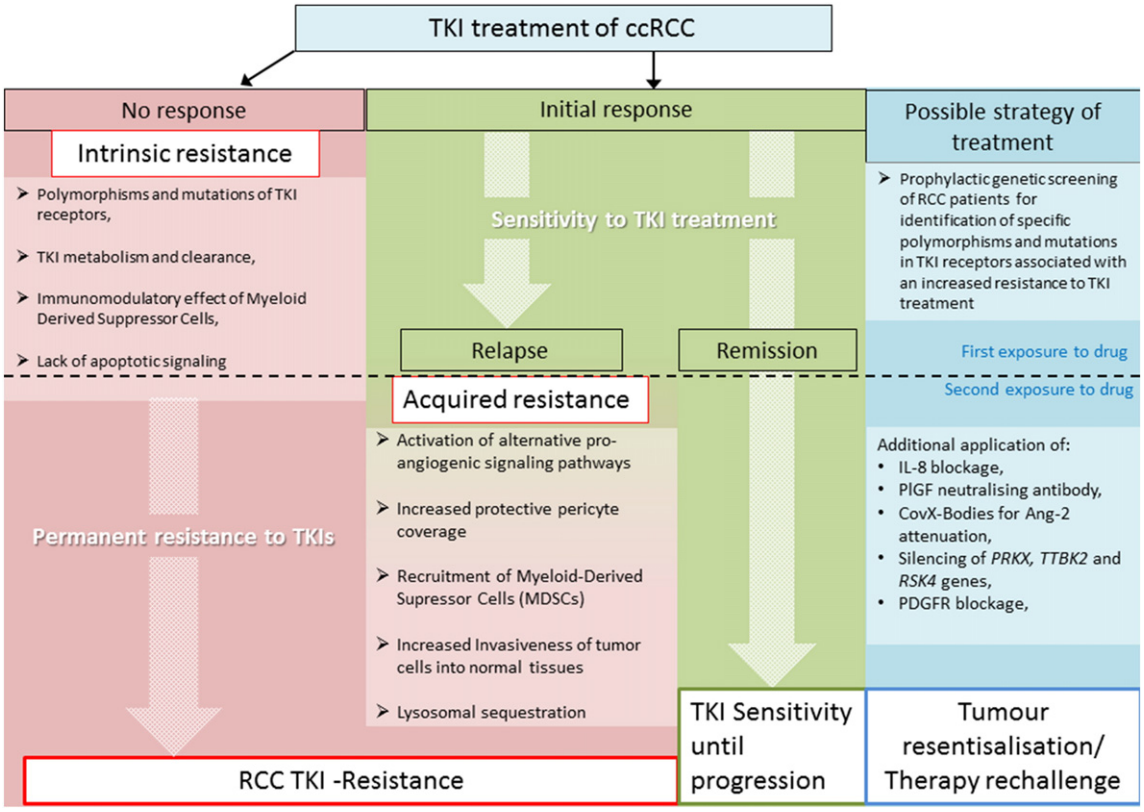

Fig. 2. The summary of TKI resistance.

pathways or single targets for pharmacological intervention. One such challenging attempt was recently presented by Bhasin et al., who created a Connectivity Map (C-Map) database providing a compendium of gene expression profiles of cancer cell lines with a relevant spectrum of bioactive molecules. Based on the altered gene expression pattern of renal cell carcinoma cell lines, they determined 3 active agents with anti-tumor activity and the potential in overcoming sunitinib mediated resistance. Clinical testing of these agents is still ongoing [125].

Finally, adequate translation of *in vitro* findings into prospective clinical trials requires establishment of a relevant and accurate *in vivo* preclinical model of carcinogenesis. Well-characterized animal models that mimic human cancers conditions are needed for several reasons: evaluation of novel drugs/drug combination efficacy and pharmacokinetics as well as the mechanisms determining drug resistance development. Considering the strong influence of the tumor microenvironment on its development *in vitro* 2D tissue cultures may not be sufficient to provide sufficient and comprehensive information about tumors. This is consistent with the fact that implantation of histologically intact RCC tumor samples was shown to more accurately represent clinical features of tumors compared to the injection of a tumor cell suspension [126–128]. Also, multiple studies have shown that the response to therapy was comparable between xenograft models and the clinical patient cases [129–131].

In conclusion, further clinical studies on anti-angiogenic drug development are needed, with an emphasis on better understanding of the potential mechanisms of resistance. Future analysis of these anti-cancer drugs combined with a molecular typing of RCC tumors in prospective clinical trials would help to address some questions related to both intrinsic and evasive resistance. Another hopeful path would be identification of reliable RCC-specific biomarkers, that will enable to predict not only the patients' response to therapy but also their predisposition for resistance development. A bench-to-bedside approach is crucial to advance the field and to improve the prognosis for recurrent RCC patients in the era of targeted, individualized therapy.

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