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Circulating Biomarkers in Advanced Renal Cell Carcinoma: Clinical Applications

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Abstract

Advances in understanding the biology of renal cell carcinoma (RCC) have resulted in treatment strategies based on molecularly targeted agents that have substantially improved the outcomes of patients with metastatic RCC. Agents targeting the vascular endothelial growth factor pathway and the mammalian target of rapamycin have shown efficacy in randomized clinical trials and received international approval for treating RCC. Multiple candidate biomarkers of the biologic activity of such targeted therapies as well as markers of treatment response and patients' prognosis are being evaluated to improve drug development and to identify patients who may obtain the greatest benefit from the various treatment options. This review summarizes recent developments in identifying circulating biomarkers of targeted therapies for metastatic RCC, including soluble proteins and circulating cells.

Keywords

blood-based biomarkers; renal cell carcinoma; prognostic markers; predictive markers; pharmacodynamic markers; resistance markers; VEGFR TKI; mTOR inhibitors

1. Introduction

Most solid lesions within the kidney are renal cell carcinomas (RCC). Clear cell RCC (ccRCC) is the most frequent, accounting for nearly 75% of the cases [1]. Among the non-clear cell RCC variants, papillary and chromophobe are the next most frequently occurring, at approximately 15% and 5%, respectively [1]. Rarer subtypes include medullary, collecting duct, mucinous tubular, spindle cell, and Xp11 translocation carcinomas [1]. These subtypes are distinguished by differences in biology [2-4], prognosis [5, 6], and treatment response [7].

In >90% of the cases, ccRCC is characterized by inactivation (via mutation, hypermethylation, and loss of heterozygosity) of the von Hippel-Lindau (*VHL*) tumor

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suppressor gene [8, 9]. Loss of VHL protein leads to accumulation of hypoxia-inducible factor alpha subunits (HIF-1 α –3 α), which heterodimerize with their binding partner ARNT (HIF-1 β) to transcriptionally regulate target genes containing hypoxia response elements. This leads to downstream upregulation of hypoxia-regulated genes, many of them proangiogenic, such as vascular endothelial growth factor (VEGF), erythropoietin, and carbonic anhydrase 9 (CA9) [10]. Although numerous genes transcriptionally activated by HIF-1 α and HIF-2 α coincide, each HIF family member is thought to also transactivate unique target sets [11]. For example, HIF-1 α has been linked to genes regulating pathways associated with glycolytic metabolism, whereas HIF-2 α is uniquely responsible for genes associated with proliferation and dedifferentiation. Identification of the resulting angiogenic phenotype of *VHL* led to the development and successful clinical application of the first targeted therapies for ccRCC, particularly of drugs targeting the VEGF and mammalian target of rapamycin (mTOR) signaling pathways [12].

The uncommon papillary type of RCC has two distinct subtypes that are associated with familial syndromes [13•, 14]. Hereditary papillary RCC (related to type 1 papillary RCC) is caused by germline mutations in the Met proto-oncogene [15], while hereditary leiomyosarcoma and RCC (HLRCC, related to type 2 papillary RCC) has been linked to mutations in the Krebs's cycle enzyme fumarate hydratase [16]. Similarly, mutations in *FLCN* (folliculin) in Birt-Hogg-Dubé syndrome primarily predispose to chromophobic RCC [17].

Complete resection remains the only curative treatment for RCC. Unfortunately, more than 30% of RCCs metastasize or recur after treatment for localized disease [18]. Since complete removal of metastatic lesions also has the potential to improve prognosis, the possibility of metastasectomy needs continuous reassessment in patients with advanced disease.

RCC is a paradigm of chemoresistant cancer. Even in combination with immunotherapy, chemotherapy is associated with low response rates [19, 20]. Because some renal tumors have induced immune responses that have even led to spontaneous regression of metastases, immunotherapeutic strategies have been tested, in some cases showing evidence of activity [21]. For more than 15 years, the established therapies for advanced RCC were interferon alfa (IFN- α) and interleukin 2 (IL-2). High-dose IL-2 produced tumor responses in 10% to 20% of patients; about 7% were complete and durable [22, 23]. However, both IFN- α and high-dose IL-2 are associated with substantial toxicity. Those and the emergence of targeted therapies have limited their use. Indeed, the currently approved VEGF and mTOR inhibitors are better tolerated and easier to administer, and have been proven to provide clinical benefit in phase III randomized clinical trials [12].

Despite these promising advances, treatment decisions in RCC still depend on exclusively clinical criteria. The emergence of molecularly targeted therapeutic options has intensified the need to identify biomarkers that more accurately determine prognosis, identify patients likely to benefit from therapy (and with which specific drug classes), and optimize doses and combination treatments, as well as allow us to understand the mechanisms underlying resistance. The benefit these agents provide, although significant, has so far been merely incremental, and resistance inevitably emerges. At this time, no molecular marker has been validated for any of these purposes. Blood biomarkers have potential important advantages: the simple, relatively inexpensive and noninvasive sample collection, making them feasible even for large multisite clinical trials and allowing us to monitor changes during treatment and disease progression, which can help identify markers of acquired resistance.

We present a concise overview of the most recent developments of blood-based biomarkers for RCC, in particular for metastatic ccRCC, and emphasize clinical applications and molecularly targeted therapies.

2. The Therapeutic Landscape in Advanced RCC

As noted above, increased understanding of the genetics and molecular biology, particularly of ccRCC, led to the development of agents targeting the VEGF and mTOR pathways. Major phase III trials have so far resulted in the regulatory approval of seven targeted agents [24-30]. Sorafenib (Nexavar, Bayer/Onyx), sunitinib (Sutent, Pfizer), pazopanib (Votrient, GlaxoSmithKline) and axitinib (Inlyta, Pfizer) inhibit the tyrosine kinases (i.e., are tyrosine kinase inhibitors, or TKIs) of VEGF receptors (VEGFRs) 1–3, among others; bevacizumab (Avastin, Genentech/Roche) specifically targets the ligand VEGF; and temsirolimus (Torisel, Wyeth/Pfizer) and everolimus (Afinitor, Novartis) affect mTOR signaling. With the exception of axitinib (which was compared with sorafenib as second-line therapy), these drugs were approved on the basis of comparisons with either cytokine therapy or placebo.

The VEGF inhibitors induce responses in up to 50% of ccRCCs, whereas the response to those that target mTOR does not exceed 10% (although this was shown in populations refractory to VEGFR TKI, in the case of everolimus, or with poor risk, as with temsirolimus) [24-30]. However, the antitumor activity of these agents, especially those targeting VEGF, is not captured solely by objective radiologic responses, because patients may obtain benefit from improved symptoms and delayed disease progression. Progression-free survival (PFS) is significantly extended with the VEGF-targeted therapies (5 to >12 months) relative to placebo or cytokines and is broadly proportional to the TKI potency of the drug (sorafenib was the first approved and is the least potent). Although the PFS associated with the mTOR-targeting agents is more modest (approximately 5 months), their front-line effect in patients with good to intermediate risk has not yet been thoroughly characterized. These drugs are also generally more active in treatment-naïve patients, and the criteria for their use in sequence remain largely empiric.

Determining the optimal dosage, dosing schedule, sequence of treatment for individual patients, and how to combine these and other agents that target alternative RCC-relevant molecular pathways is being addressed in multiple ongoing studies.

3. Clinical Applications for Circulating Biomarkers in Advanced RCC

Biomarkers indicate important clinical events such as cancer onset, recurrence, progression, or patient death and can be used for risk assessment, screening, diagnosis, prognosis, prediction of benefit from specific therapies, and evaluation of recurrence or progression of disease [31]. Prognostic factors relate to a disease's natural history independently of therapy, including the risk of disease occurrence, relapse, progression, or survival. Predictive markers identify patients who will experience different degrees of benefit from a specific therapy or class of related therapies, thereby permitting individualized therapy with maximum benefit and minimum risk.

Randomized clinical trials that use a comparator treatment group or groups are typically required to declare a marker as predictive. If no comparator is available, as in single-arm studies, markers simply indicate association with clinical benefit, because it is not possible to know whether the marker is prognostic, predictive, or both for the intervention being used. Pharmacodynamic or markers of activity assess the expected biologic activity of a drug, reflecting the effects on the tumor or a surrogate tissue, with or without correlation with drug concentration or clinical benefit. The next sections review recent progress in soluble and cellular blood markers for targeted therapies in advanced RCC.

3.1. Soluble Biomarkers

3.1.1. Prognosis—In RCC, as in many other solid tumors, initial attempts to predict patient outcome focused on individual clinical features. Several prognostic models have established clinical parameters that identify patient characteristics associated with survival outcomes [32•, 33, 34•], reflecting the variable natural history of metastatic RCC irrespective of treatment. Antiangiogenic therapy–induced toxicity has also been studied. Most notably, the development of hypertension is solidly associated with clinical outcome in patients with metastatic RCC treated with different VEGF inhibitors [35•, 36]. However, until the biologic determinants of this response are well defined and surrogates of its development can be used before rather than during treatment, the clinical value of hypertension as a marker of clinical benefit from VEGF-targeted therapy remains limited.

Analyses of *VHL* mutational status in ccRCC have yielded inconsistent data to support its value as either prognostic or predictive (see ref. [37•] for a recent review). The activation status of the HIF subunits [38] and multiple HIF-responsive genes are currently being examined. One HIF target, VEGF, and other angiogenesis-related and tumorigenic factors in serum or plasma have been evaluated in multiple clinical trials of targeted agents in RCC. VEGF is important not only in physiologic and tumor-related angiogenesis but also in processes leading to tumor growth, survival, and metastasis [39]. The VEGF family comprises several VEGF proteins (A–F, although “VEGF” designates VEGF-A) and placental growth factor. VEGF, which binds to two receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, flk-1), is considered the most important regulatory factor of tumor angiogenesis. Naturally occurring soluble (s) forms of VEGFR-1, VEGFR-2, and VEGFR-3 (which is involved in lymphangiogenesis) are detectable in peripheral blood [40–42]. Patients who have RCC and relatively high VEGF concentrations typically have higher tumor stage and grade and worse performance status and overall prognosis [25, 43, 44•, 45].

In the phase III TARGET trial of sorafenib versus placebo in patients with advanced ccRCC previously treated with cytokine therapy, baseline VEGF concentrations correlated with PFS ($P=0.0231$) and OS ($P=0.0416$) in multivariate analyses of placebo-treated patients and with short OS ($P=0.0145$) in multivariate analysis of sorafenib-treated patients [25]. Results were similar the AVOREN phase III trial of bevacizumab plus IFN versus IFN plus placebo, in which high baseline VEGF concentrations correlated with shorter PFS in both arms [46].

Follow-up analysis of the TARGET trial results evaluated VEGF, sVEGFR-2, sCA9, tissue inhibitor of metalloproteinase-1 (TIMP-1), and Ras p21 in plasma [44•]. TIMP-1 remained prognostic for survival ($P=0.002$) in a multivariate analysis model that also included performance status score, MSKCC prognostic score, and other biomarkers.

Several groups have identified IL-6 as a marker of prognosis in patients with metastatic RCC [43, 47, 48]. Aggressive forms of RCC are associated with a paraneoplastic syndrome characterized by increased expression of inflammatory cytokines, including IL-6 [49, 50], which may be important in cancer progression through autocrine or paracrine mechanisms. Negrier et al. [43] reported that high concentrations of IL-6 significantly correlated with shorter PFS and overall survival (OS) and was an independent determinant of OS in RCC patients receiving IL-2 or IFN therapy. A separate pro-metastatic marker, osteopontin (OPN), is relatively high in patients with disseminated RCC, and high concentrations are also associated with poor survival [51].

In a different study [52•], we assessed the prognostic significance of seven cytokines and angiogenic factors (IL-6, IL-8, hepatocyte growth factor [HGF], OPN, VEGF, TIMP-1, and E-selectin) derived from initial screening for markers of clinical benefit from pazopanib in a

separate phase II study [53] and the placebo-controlled phase III randomized trial of pazopanib in advanced RCC [26]. By using a Clinical Laboratory Improvement Amendments–certified multiplex ELISA platform, we found that high concentrations of IL-6 ($P<0.001$), IL-8 ($P<0.002$), and OPN ($P<0.001$) correlated with poor prognosis for PFS in the placebo arm [52•].

Thus, besides VEGF, these studies established TIMP-1, IL-6, IL-8 and OPN as candidate prognostic markers for patients with advanced RCC. One or more of these markers has potential to enhance the prognostic ability of currently used clinical schemes.

3.1.2. Prediction—Predictive markers are critically important in oncology, particularly in situations like advanced ccRCC, in which multiple treatment options with different mechanisms of action and activity and toxicity profiles exist, and what sequence to use for individual patients remains undefined. Unfortunately, no marker in blood has validated predictive ability, although recent studies demonstrate early progress for VEGF inhibitors.

In the phase III TARGET trial of sorafenib versus placebo, patients with the highest concentration of VEGF obtained greater relative benefit from the VEGFR TKI sorafenib than did those with lower concentrations [25]. However, other studies, like AVOREN (the anti-VEGF antibody bevacizumab plus IFN versus IFN plus placebo), yielded inconsistent results, perhaps because bevacizumab was tested in combination [46].

Other studies showed that expression of clusters of biologically related molecular factors often correlate in cancer patients [54•, 55•, 56]. For example, we recently found that a six-marker angiogenic signature comprising OPN, VEGF, sCA9, collagen IV, sVEGFR-2, and tumor necrosis factor–related apoptosis-inducing ligand identified patients who obtained greater PFS benefit from sorafenib alone than from sorafenib plus IFN [54•].

Similarly, we analyzed circulating factors predictive of different PFS benefit from pazopanib treatment versus placebo in the phase III study and found only IL-6 to be predictive ($P=0.009$), with hazard ratios of 0.31 (95% CI=0.21–0.44) in the high IL-6 group and 0.55 (95% CI=0.38–0.81) in the low group. Placebo-treated patients with high IL-6 had dramatically shorter PFS than those with low IL-6 (9.9 vs 24 weeks; $P<0.0001$), but the IL-6 groups did not differ significantly in the pazopanib arm (32.6 vs 42.3 weeks) [52•]. Thus, high IL-6 concentration predicted greater benefit from pazopanib than from placebo, suggesting that the negative prognostic effect of high IL-6 was attenuated by pazopanib. High concentrations of six angiogenic and immunomodulatory factors in this analysis, IL-6, IL-8, HGF, OPN, VEGF, and TIMP-1, demonstrated an intriguing negative effect on OS plus a greater relative OS benefit from pazopanib [57].

Together, these preliminary results suggest that patients with metastatic RCC and elevated expression of angiogenic factors obtain greater relative benefit from VEGF-targeted therapies. Of relevance, we recently identified two groups of patients with metastatic ccRCC characterized by their expression of alternative inflammatory or angiogenic circulating factors [54•].

3.1.3. Biologic Activity—Soluble factors have also been investigated as biomarkers of biologic activity of VEGF inhibitors. Consistent with their mechanisms of action, VEGF and placental growth factor increase and sVEGFR-2 and sVEGFR-3 decrease during treatment with VEGFR TKIs [42, 58, 59]. Generally, larger changes occur in patients experiencing treatment response [42], but the extent of factor modulation varies widely among individuals and depends on the specific drug, its potency, and its profile of molecular targets inhibited, which is broad for many available drugs.

Single nucleotide polymorphisms (SNPs) are genetic determinants of drug exposure in the host typically analyzed with germline DNA from peripheral blood. SNPs have implications for drug selection, dosage and/or schedule optimization, and early intervention for specific toxicity. Two very recent studies have established germline SNP variants in angiogenesis- and exposure-related genes associated with response and tolerability to pazopanib and sunitinib in patients with advanced RCC [60•, 61•].

3.1.4. Treatment Resistance—Similar to the way a wide variety of anticancer agents such as chemotherapy [62] can induce responses other than those intended, angiogenesis inhibitors may induce multiple off-target tumor cell growth-promoting cytokines, chemokines, and growth factors that may actually facilitate tumor progression and decrease therapeutic response in unintended ways, as suggested by preclinical studies [63, 64]. These changes in circulating factors are mediated in large part by the host rather than the tumor [55•, 65, 66•].

A glimpse of the pharmacologic complexity of the systemic response to “broad-spectrum” VEGFR TKIs came from simultaneous profiling of more than 50 cytokines and angiogenic factors in plasma of patients with metastatic ccRCC treated with sorafenib or sorafenib plus IFN [54•]. As expected, sorafenib induced reciprocal changes in VEGF and sVEGFR-2 concentrations, but several other angiogenic mediators were significantly modulated, effects that were blunted (sVEGFR-2, VEGF, collagen IV, E-selectin) or enhanced (monocyte chemotactic protein 1, IL-18) by the addition of IFN. Prominent changes also occurred in several helper T cell type 2 (IL-5, IL-6, IL-10, IL-13) and type 1 (IL-12 p40, IFN- γ) and hematopoietic (macrophage colony-stimulating factor 1, growth-regulated alpha protein) cytokines.

Some candidate molecular factors implicated in resistance to VEGF inhibitors to date pending clinical validation include IL-8 and HGF [67•, 68•].

3.2. Cellular Biomarkers

3.2.1. Circulating Endothelial Cells (CECs) and Progenitors (CEPs)—CECs and bone marrow-derived CEPs are leatively higher in the circulation of patients with metastatic cancer and, in some cases, are associated with prognosis [69, 70]. Differentiated (“mature”) CECs arise from blood vessel walls; their numbers increase after vascular damage [71, 72]. CEPs may contribute to neovascularization by releasing proangiogenic cytokines and by being incorporated into and stabilizing nascent blood vessels [73]. In humans, mature CECs and CEPs can be distinguished on multicolor flow cytometry from other circulating cells on the basis of surface markers.

Endothelial cells express molecular targets, including the VEGFRs, for the angiogenesis inhibitors currently used for treatment of advanced RCC. The CEPs are particularly known to mobilize in response to VEGF and other angiogenic mediators during tumor progression or as a result of vascular disruption, antiangiogenic treatments, or chemotherapy [74, 75]. CECs may therefore be a pharmacodynamic marker of treatment benefit and prognosis.

Different investigators have reported that patients with sporadic RCC have more CECs than healthy control subjects do [76-78], although the numbers may not differ between primary and metastatic disease [79]. Numbers of CEPs are higher in patients with VHL disease who have developed RCC than they are in those with manifestations of VHL with tumors other than RCCs [76]. CEPs decrease after nephrectomy in patients with nonmetastatic RCC [76], suggesting that enumerating CEC subsets, particularly CEPs, could be a means of surveillance in patients at risk for developing RCC.

In one series of 22 patients with primary sporadic RCC who had undergone nephrectomy, the CEP:mature CEC ratio (a candidate marker of host angiogenic drive) was higher in patients with recurrent disease than it was in those without recurrence [79], indicating that increasing numbers of CEPs postoperatively may portend higher risk for recurrent RCC. Moreover, patients with metastatic RCC and higher counts of a specific progenitor subpopulation (CD45^{dim}CD34⁺VEGFR-2⁺) appeared to have a worse prognosis [80].

Our group predicted that effective VEGFR inhibition would result in decreased CEPs, by inhibiting their mobilization from the bone marrow, and increased sloughed vessel wall-derived CECs [81]. Indeed, several exploratory studies showed that in the context of VEGFR TKI therapy in patients with metastatic RCC, the number of CECs may change during treatment in correlation with measures of clinical benefit, such as PFS and response [77, 78, 82]. These and studies in other cancer types illustrated that CEC numbers change dynamically with angiogenesis inhibition and that the magnitude and direction of the change, as well as the specific CEC subpopulation that changes, are important parameters to consider.

Unfortunately, although CECs could be uniquely valuable for managing ccRCC, the difficulty in quantifying them reliably (owing to their very low numbers in the circulation and lack of entirely specific markers for their detection, which demands multiparametric analyses) has decreased the early enthusiasm for their use as a biomarker.

3.2.2. Cells of Hematopoietic Lineage—Besides CEPs, certain myeloid cell types, including monocyte and neutrophil precursors, macrophages, mast cells, dendritic cell precursors, and the so-called myeloid-derived suppressor cells (MDSCs), may contribute to tumor progression by facilitating angiogenesis, immunosuppression, and metastasis [83, 84]. Such cells can secrete growth factors, remodel extracellular matrices (promoting tumor cell motility and invasiveness), provide structural support to adjacent endothelial cells, impair host antitumor adaptive immunity, and establish niches to facilitate the homing and target-organ colonization of disseminated tumor cells. Some of these properties could also be involved in resistance to targeted antiangiogenic therapies [85-88].

As a consequence of high concentrations of VEGF, IL-6, and other still not well-characterized tumor-derived factors, peripheral blood from patients with advanced RCC may contain relatively high numbers of immature myeloid cells that have morphologic features of granulocytes and can impair T-cell function, behaving like MDSCs [87•, 88, 89]. High expression of CD66b, CD11b, and VEGFR-1 characterizes these cells [87•, 90]. Of interest, a high neutrophil count has been associated with poor prognosis in patients with metastatic RCC treated with VEGF-targeted agents [32••].

Although the anti-VEGF antibody bevacizumab does not appear to affect MDSC levels in peripheral blood [87•], the VEGFR TKI sunitinib has direct anti-MDSC activity [91, 92]. In contrast to sorafenib, sunitinib can also reduce numbers of circulating regulatory T cells without reducing the function of dendritic cells [93]. However, these immunomodulatory properties seem to be independent of sunitinib's antitumor effect [91], making their value as possible biomarkers of sunitinib treatment benefit unclear.

Still, exploratory studies of sunitinib have revealed significant correlations between changes in specific myeloid dendritic cell subsets and regulatory T cells and improved objective responses, PFS, and OS in patients with metastatic RCC [94, 95].

3.2.3. Circulating Tumor Cells (CTCs)—CTCs are commonly present in very low numbers in the blood of patients with solid tumors, even those with metastases. This

necessitates using tumor-specific markers to detect CTCs and enrichment methods (e.g., immunomagnetic techniques, flow cytometry, density gradients, mechanical filters, microchips) to capture them. Despite considerable interest in utilizing CTCs as biomarkers in RCC, efforts to reliably detect RCC cells in blood have been hindered by the general absence of markers that are widely and specifically expressed in RCC cells relative to background blood cells. Epithelial markers such as the epithelial cell adhesion molecule and cytokeratins are useful for differentiating most CTCs in patients with other common solid tumors, but RCC cells often lack epithelial differentiation. It is unsurprising that very few reports covering this have been published.

One such study evaluated the prognostic significance of CTCs in 154 patients with RCC by using cell density and immunomagnetic enrichment as well as cytokeratin (CK) 8/18 for detection [96]. Two kinds of putative CTCs were detected: CK⁺ and large CK⁻ hemalaun-blue-staining (BI⁺ cells) hematopoietic lineage-negative tumorlike cells. Peripheral blood specimens from only 4.5% of the patients had CK⁺ cells, whereas 38% had large BI⁺ cells. Cell numbers ranged from 1 to 51 (mean, 6 cells) and directly correlated with the presence of lymph node and distant metastases.

The low expression of CK by CTCs in patients with RCC was confirmed in a second study that used the U.S. Food and Drug Administration-approved CellSearch platform in 25 patients with metastatic RCC [97]: CTCs were found in only 16% of those patients.

Clearly, new technical developments are necessary before CTC analysis can become a useful biomarker for RCC patients.

4. Conclusions

Although several promising blood biomarkers are now available, particularly for angiogenesis inhibitors, additional progress is needed to more accurately estimate prognosis and likelihood of response and resistance to therapy in individual RCC patients. New molecularly targeted agents that will likely be combined with the available therapies are on the immediate horizon. Significant progress will require simultaneous analysis of multiple aspects of RCC biology in blood and tissue for identifying and optimizing biomarkers in efficient exploratory studies, followed by validation in prospective randomized clinical trials to facilitate their clinical implementation.

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