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Molecular Cancer Therapeutics

Review

Breaking through a Plateau in Renal Cell Carcinoma Therapeutics: Development and Incorporation of Biomarkers

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Abstract

With the Food and Drug Administration approval of 6 novel targeted agents since December 2005 and limited comparative trials to discern relative efficacy, the treatment of metastatic renal cell carcinoma (RCC) has become immensely complex. The research community must look to novel ways in which to identify appropriate candidates for selected targeted therapies; one potential strategy is the use of clinical and molecular biomarkers. A growing body of knowledge-related von Hippel Lindau-driven pathways in this disease has highlighted the potential role of hypoxia-inducible factor subtypes in distinguishing RCC patients clinically. Techniques applied in other malignancies, such as gene expression and proteomic profiling, may also ultimately allow for clinical stratification. An emerging understanding of immunologic phenomena that may affect cancer progression (i.e., tumor infiltration by CD68 lymphocytes, memory T-cells, etc.) has unveiled a number of other potential biomarkers of response. Several vascular endothelial growth factor receptor-directed therapies classically thought to function as antiangiogenics may also have complex effects upon the tumor microenvironment including the associated immune cell milieu. As such, immunologic parameters could potentially predict response to current therapies. Finally, clinical biomarkers, such as hypertension, may predict the efficacy of several currently available targeted agents, although implementation of such biomarkers remains challenging. Herein, the clinical relevance of putative RCC biomarkers is examined in detail. *Mol Cancer Ther; 9(12); 1–11. ©2010 AACR.*

Introduction

Over the past decade, the clinical and scientific community has witnessed unprecedented developments in targeted therapies for metastatic renal cell carcinoma (mRCC). Whereas the oncologist was previously equipped with a limited number of immunotherapeutic agents, such as interferon-α (IFN-α) and interleukin-2 (IL-2), a total of 6 targeted agents have been approved by the US Food and Drug Administration within the past 5 years (1). These agents can be broadly divided into 2 categories: (1) agents that directly counteract vascular endothelial growth factor (VEGF)-mediated signaling and (2) agents that act on the mammalian target of rapamycin (mTOR) pathway. Agents in the former category supported by randomized, phase III data include sunitinib, sorafenib, pazopanib, and bevacizumab (2–6). Similarly, mTOR inhibitors supported by phase III data in mRCC include temsirolimus and everolimus (7, 8).

Oncologists are now charged with determining which targeted agent to select for the patient with mRCC. Limited comparative trials are available to juxtapose efficacy; thus, the decision is typically based on patient preference after a thorough discussion of benefits, adverse effects associated with each agent and consideration of comorbidities. Borrowing paradigms from other diseases, it is likely that targeted agents are more apt to work in circumstances in which the biological target is more abundant. For instance, in breast cancer, HER2-directed therapies have shown profound clinical benefits specifically in the subset of patients with HER2 overexpression (9–12). As a more recent example, the oral anaplastic lymphoma kinase (ALK) inhibitor, crizotinib, appears to have significant activity in patients with non–small cell lung cancer who bear the *EML4-ALK* fusion oncogene (13). Similarly, certain patients with melanoma may be exquisitely sensitive to the agent PLX4032, targeting a frequently mutated form of the serine–threonine kinase B-RAF (*BRAF* V600E; ref. 14). In a similar fashion, identification of relevant biomarkers in RCC may refine current algorithms for treatment selection. Herein, we review the current status of biomarkers in RCC in hopes

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of developing a framework for their use as prognostic and predictive tools.

Hypoxia-Inducible Factor-α and Related Pathways

A key driver in RCC tumorigenesis is the von Hippel Lindau (VHL) gene mutation. *VHL* encodes a tumor suppressor protein (pVHL) with a molecular weight between 24 and 30 kDa (15). In its native form, pVHL typically forms multimeric complex with several other moieties (Elongin B, Elongin C, Cul2, and Rbx1) and binds to hypoxia inducible factor-α (HIF-α) in the setting of hypoxia (16–18). Purified pVHL appears to have ubiquitin ligase activity, directing HIF-α toward proteasomal degradation (19). Mutation or hypermethylation of *VHL* occurs in up to 80% of sporadic clear cell RCC (ccRCC) cases; these phenomena may interrupt formation of the pVHL complex and thereby stabilize HIF-α (20, 21). In addition, HIF-1α levels may also be upregulated by oncogenic signaling through signal transducer and activator of transcription 3 (STAT3), commonly activated in human tumors such as RCC (22, 23). The resultant effect is increased binding of HIF-α to hypoxia response elements, leading to transcription of HIF target genes such as *VEGF* (24) or carbonic anhydrase IX *(CAIX*; ref. 25). These observations offer mechanistic rationale for agents that target the VEGF-signaling axis in this disease.

Recognition of 2 subtypes of HIF-α (HIF-1α and HIF-2α) has led to the development of a potential biomarker for ccRCC. These 2 subunits can form complexes with a β subunit, allowing for subsequent binding to HIF response elements. HIF-1α and HIF-2α differ with respect to their sites of expression. Whereas the former is ubiquitously expressed, the latter is expressed principally in endothelium, heart, lungs, kidney, and small intestine (26–28). In *in vivo* assays, only HIF-2α was able to overcome pVHL-induced suppression (29, 30). This phenomenon may be a consequence of HIF-2α–mediated increases in c-myc activity, documented in recent *in vitro* studies (31).

A comprehensive analysis of 57 sporadic human ccRCC specimens by Gordan et al. has elicited a potential classification schema on the basis of HIF subtype (32). In this cohort of patients, only 12% were *VHL* wild-type with no detectable level of HIF-α. The remainder bore *VHL* mutations and had either increased expression of both HIF-1α and HIF-2α (termed the H1H2 phenotype, 61%), or HIF-2α alone (termed the H2 phenotype, 27%). Consistent with previously noted observations, H2 tumors exhibited greater expression of *c-myc*–activated targets such as cyclin D2 and E2F. Furthermore, H2 tumors exhibited increased expression of homologous recombination mediators BRCA1, BARD21, and XRCC2 (notably, homologous recombination allows DNA-damaged cells to avoid checkpoint activation).

The expression profile of H1H2 tumors appears to be markedly different than the other subtypes (31, 32). In H1H2 tumors, increased expression of the growth factor signaling molecules Akt2 and RhoC was observed. Furthermore, increased phospho-S6 and phospho-ERK was seen in both wild-type and H1H2 tumors. The findings of Gordan et al. thus suggest several new targets for ccRCC therapy. Furthermore, they suggest a potential stratification schema for determining the most appropriate candidates for specific therapies. Albeit speculative, increased Akt2 and phospho-S6 activation in H1H2 tumors may suggest a potential role for the mTOR inhibitors, everolimus and temsirolimus, and perhaps a benefit from VEGF-tyrosine kinase inhibitors (VEGF-TKI) that abrogate upstream signaling. Furthermore, the unique expression profile of H2 tumors may lead to an intrinsic resistance to VEGF-directed therapies—for this subset of patients, further assessment of agents that interrupt DNA repair pathways (i.e., PARP inhibitors) and *c-myc*–mediated signaling may be prudent.

Gene Expression Profiling

Rini et al. have recently presented the largest and most comprehensive gene profiling effort in RCC to date (33). Utilizing tissue specimens derived from a cohort of 931 patients with stage I–III ccRCC, RT-PCR was used to quantify RNA expression of 732 candidate genes. Clinical follow-up was available for a median of 5.6 years in this cohort, and Cox models were used to determine which genes were associated with recurrence-free interval (RFI). Notably, several clinicopathologic variables were associated with RFI, including clinical stage, Fuhrman grade, size, and lymph node involvement ($P < 0.001$ for each; ref. 34). Of the 732 genes assessed, 448 genes were associated with RFI ($P < 0.05$). On further multivariate analysis accounting for clinicopathologic covariates and false discovery rates, a total of 16 genes remained associated with RFI. Among these genes are several with a plausible relationship to RCC tumorigenesis-–for instance, *EMCN* and *NOS3* are associated with angiogenic pathways, whereas *CCL5* and *CXCL9* are associated with immune-related pathways. The 16 candidate genes (along with 5 reference genes) will be evaluated in distinct validation cohorts.

Dalgliesh et al. have used a broader sequencing approach in a smaller cohort of RCC patients to identify several moieties intricately linked to RCC biology (35). In 101 ccRCC specimens, the group sequenced 3,544 protein-encoding genes. A total of 5 genes with relevance to cancer development were identified that exhibited clustering of somatic mutations (*SETD2*, *JARIDI1C*, *NF2*, *UTX*, and *MLL2*). Subsequent focused screening of a larger cohort including 407 ccRCC specimens demonstrated inactivating mutations in the genes encoding SETD2 (a histone H3lysine 36 methyltransferase) and JARID1C (a histone H3 lysine 4 demethylase). These thorough experiments strongly implicate the role of these histone-modifying genes in RCC pathogenesis. *NF2* gene mutations were also found in secondary screening; importantly, mutations in this gene were found

independently of *VHL* mutation or expression of a hypoxia expression phenotype. This suggests that *NF2* mutation (associated with neurofibromatosis II and the formation of acoustic neuromas and meningiomas) may also define a distinct subset of ccRCC with unique pathogenesis.

The information rendered from traditional gene profiling techniques can be augmented through the additional knowledge provided by broad transcriptomic profiles. Cifola et al. have done a detailed analysis of 27 RCC samples (36). DNA copy number alterations and loss of heterozygosity events were determined using a single-nucleotide polymorphism (SNP) array technology. Outside of previously recognized areas of DNA alteration, the genome-wide map highlighted several new sites of deletion, amplification, and loss of heterozygosity (36). A simultaneous assessment of the transcriptomic profile of these specimens suggested that 27 differentially expressed genes were present in DNA-amplified regions. For instance, the transcripts encoding lysyl oxidase (*LOX*, 5q) and chemokine C-X-C receptor 4 (CXCR4, 2q) were noted to be upregulated—notably, the associated regions of DNA were concomitantly amplified. Both LOX and CXCR4 may play a critical role in the metastatic potential of tumor, possibly promoting tumor cell trafficking into premetastatic niches (as suggested in murine studies; refs. 37–40). Ultimately, paired genomic and transcriptomic profiling may render unique targets for RCC therapy and may offer individualized prognostic information.

A greater understanding of epigenetic phenomena may also allow for distinct characterization of renal tumors. A panel of 18 cancer-related genes was assessed for methylation status in a pool of 85 resected RCC specimens and 62 paired normal tissue samples (41). Notably, the genes fell into one of several groups, including those involved in cellular communication, nucleic acid metabolism, signal transduction, energy regulation, and cell-cycle progression. Altered methylation patterns were observed in *CDH1* and *RASSF1A*, involved in cellular communication and cell-cycle progression, respectively. Differences in methylation patterns were also noted between clear cell and non–clear cell RCC specimens—for instance, *PTGS2* methylation (involved in metabolic pathways) was increased in clear cell as opposed to papillary RCC. In a similar but separate series of experiments, 38 specimens derived from nephrectomy in RCC patients (and paired normal tissue) were assessed for methylation of 19 genes that were noted to be underexpressed in RCC (42). A total of 5 genes were found to be hypermethylated, and among these were 2 genes encoding tumor suppressor proteins (TFAP2A and MT1G). These epigenetic phenomena may, therefore, highlight characteristic features of RCC specimens, and if validated in larger cohorts, could serve as critical prognostic or predictive tools.

Broader approaches across multiple cancer subtypes may also cause genetic alterations that more uniformly affect cancer growth and proliferation across histologies. As one example, Beroukhim et al. have assessed 3,131 cancer specimens across 26 histologic subtypes (including RCC; ref. 43). Through high-resolution analyses of somatic copy number alterations, it was found that *BCL2*-related genes (functioning in apoptosis regulation) and genes encoding moieties along the NF-κB signaling pathway were enriched. Two amplifications peaks were seen for the *BCL2*-related genes *MCL1* and *BCL2L1*. Subsequent experiments comparing *MCL1*-amplified and nonamplified cell lines showed greater growth inhibition in amplified cell lines treated with *MCL-1* shRNA. Similar results were yielded in experiments assessing *BCL2L1* inhibition in several models.

Proteomic Profiling

In comparison to gene expression profiling, the amount of published data on renal tumor proteomic profiles is less abundant. Nonetheless, several emerging datasets suggest the potential utility of this tool. In one series of experiments, serum samples from 65 patients with RCC were compared with 34 patients affected with benign renal tumors and 69 normal controls (44). Using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), a total of 29 proteins with differential expression were identified between patients with RCC and normal controls. A lesser number of proteins, 18, were identified that differed between RCC patients and those with benign tumors. Using a diagnostic decision tree based on the protein expression profile, this study suggested that SELDI-TOF-MS could separate RCC patients from normal controls with an appreciable sensitivity and specificity of 81.8% and 100%, respectively. A separate report from the same group suggested the utility of adding CT scans to proteomic evaluation, enhancing the diagnostic capability of the test (45).

The *VHL*-driven biology of RCC may ultimately be assessable through proteomic techniques. In a recent report, Aggelis et al. used the *VHL*-defective UMRC2 RCC cell line (46). Cells were transfected with either vector control or *VHL* cDNA. Membrane proteins were enriched and assessed using SDS-PAGE coupled to liquid chromatography mass spectrometry; analysis showed 19 differentially expressed proteins. These proteins included several moieties relevant to VHL-dependent pathways, including transferrin receptor-1 and α3β1 integrin (47, 48). Other novel protein markers were defined, including CD166 (or activated leukocyte cell adhesion molecule) and CD147 (or extracellular matrix metalloproteinase inducer), both of which were upregulated in *VHL*-defective cells. In a small cohort of matched patient specimens ($n = 8$), CD166 expression was observed to be higher in tumor tissue than in normal tissue.

Pairing genomic and proteomic profiles may provide the most extensive pool of information for the individual patient, although combining these technologies is complex.

In one representative study, malignant and matched normal tissue was obtained from 37 patients with RCC who had undergone nephrectomy (49). Standard 2D-PAGE proteomic techniques identified 334 differentially expressed proteins. In contrast, PROTEOMEX assessment (proteomic techniques paired with serologic testing to identify immunogenic biomarkers) identified 50 proteins, whereas transcription profiling identified 110 differentially transcribed genes. In combination, only 3 candidate biomarkers were elicited (ANXA4, tubulin α-1A chain and UCHL1). Nonetheless, with greater refinement of each technique and larger scale validation, combined genetic and proteomic profiling may represent a novel approach to characterizing patients with RCC. Larger scale validation of these techniques will improve the signal–noise ratio observed in these early studies. As one example of this, the Cancer Genome Atlas is a 5-year NIH-funded effort currently devoting attention to comprehensive genomic assessment of 20 cancer types (50). Amongst the urologic tumors under study, the Cancer Genome Atlas will focus on both conventional and papillary RCC.

Immunologic Markers

Tumors contain a number of immune cells that can positively or negatively influence their progression. Tumor infiltration by CD8 lymphocytes and especially memory T cells are considered a strong indicator of disease-free survival as shown by large cohort studies of various solid cancers including RCC (51, 52). In contrast, the presence of tumor-infiltrating regulatory T cells (T_{regs}) and myeloid cells, such as tumor-associated macrophages and myeloid-derived suppressor cells (MDSC), is known to be associated with tumor immune evasion and reduced survival (51, 53, 54). The elimination of immunosuppressive activity by antibody-mediated T_{regs} depletion or blockade of T-cell–imparing molecules expressed on tumor cells, such as B7-H1 and B7-H4, are potential strategies for RCC therapy (55, 56). In addition, whereas the putative mechanism of most antiangiogenic agents is principally antagonism of the VEGF-signaling axis, there is an emerging understanding that these agents may also elicit their antitumor effect through complex immunologic phenomena (Fig. 1). As one example, sunitinib has been shown to reduce recruitment of MDSCs and tumor-infiltrating T_{regs} at the site of tumor, thereby augmenting the antitumor immune response (57). The effect on MDSC recruitment appears to be mediated by direct inhibition of STAT3 in MDSCs by sunitinib.

Clinical support for these findings is provided by Finke et al. (58). In a series of 42 patients with mRCC treated with sunitinib monotherapy, serial blood collections were done on days 1 and 28 of therapy. Sunitinib was dosed on a standard 4-week on, 2-week off schedule at a dose of 50 mg oral daily. In comparison to age-matched blood from normal donors, patients treated with sunitinib had an improved type 1 T-cell cytokine response (increased IFN-γ producing T-cells and reduced IL-4 production) with sunitinib therapy, but had a decrease in circulating T_{regs}. The prognostic value of T_{regs} in RCC has been established in a separate study. When assessed alongside clinicopathologic criteria in 125 patients with RCC, multivariate analysis indicated that an increase in peritumoral T_{regs} along with higher TNM stage, higher tumor size, and higher nuclear grade were each independent predictors for shorter overall survival (OS) and RFI. A predictive role of T_{regs} in the context of sunitinib therapy has yet to be established.

Other immune effector cells have been explored as predictors of sunitinib response. Myeloid differentiation was assessed in a cohort of 26 patients with advanced RCC treated with sunitinib therapy on a standard schedule, as in the previous study (59). With measurements at baseline, at 28 and 42 days in the first treatment cycle, it was observed that higher levels of $CD1c^{+}$ myeloid dendritic cells predicted improved progression-free survival (PFS) with sunitinib. The effect of sunitinib on cells of the innate immune system has been assessed in a similar fashion; however, preliminary reports suggest no change in natural killer cell populations with sunitinib therapy (60).

Do the sunitinib-induced changes in immune phenotype represent a class effect among VEGF-directed therapies? Albeit limited, there is evidence to the contrary. One such study juxtaposed the activity of sorafenib and sunitinib in C57BL/6 mice immunized with OVA257–264 peptide (61). Although the proportion of T_{regs} decreased over the duration of therapy with sunitinib, this phenomenon was not observed with sorafenib. Furthermore, sorafenib (unlike sunitinib) inhibited the ability of mice to mount an antigen-specific T-cell response. Thus, in moving forward, it will be important to discern the immunologic effect of each targeted agent independently. Monitoring these effects may have a prognostic or predictive role.

Clinical Biomarkers

Several prognostic schema [i.e, the UCLA Integrated Staging System and Memorial Sloane Kettering Cancer Center (MSKCC) criteria] have been devised and validated in large cohorts of patients with both advanced and localized mRCC (62–69). However, these were largely formulated during the era of cytokine therapies. Given the rapid shift toward newer targeted therapies, a revised model has been suggested. Using clinical data from 645 patients with mRCC who received first line with VEGF-directed agents, Heng et al. employed Cox proportional hazards regression and bootstrap validation to identify independent prognostic factors for OS (70). Incorporating several of the original MSKCC criteria, the revised model adds platelet and neutrophil counts to establish favorable, intermediate and poor risk groups.

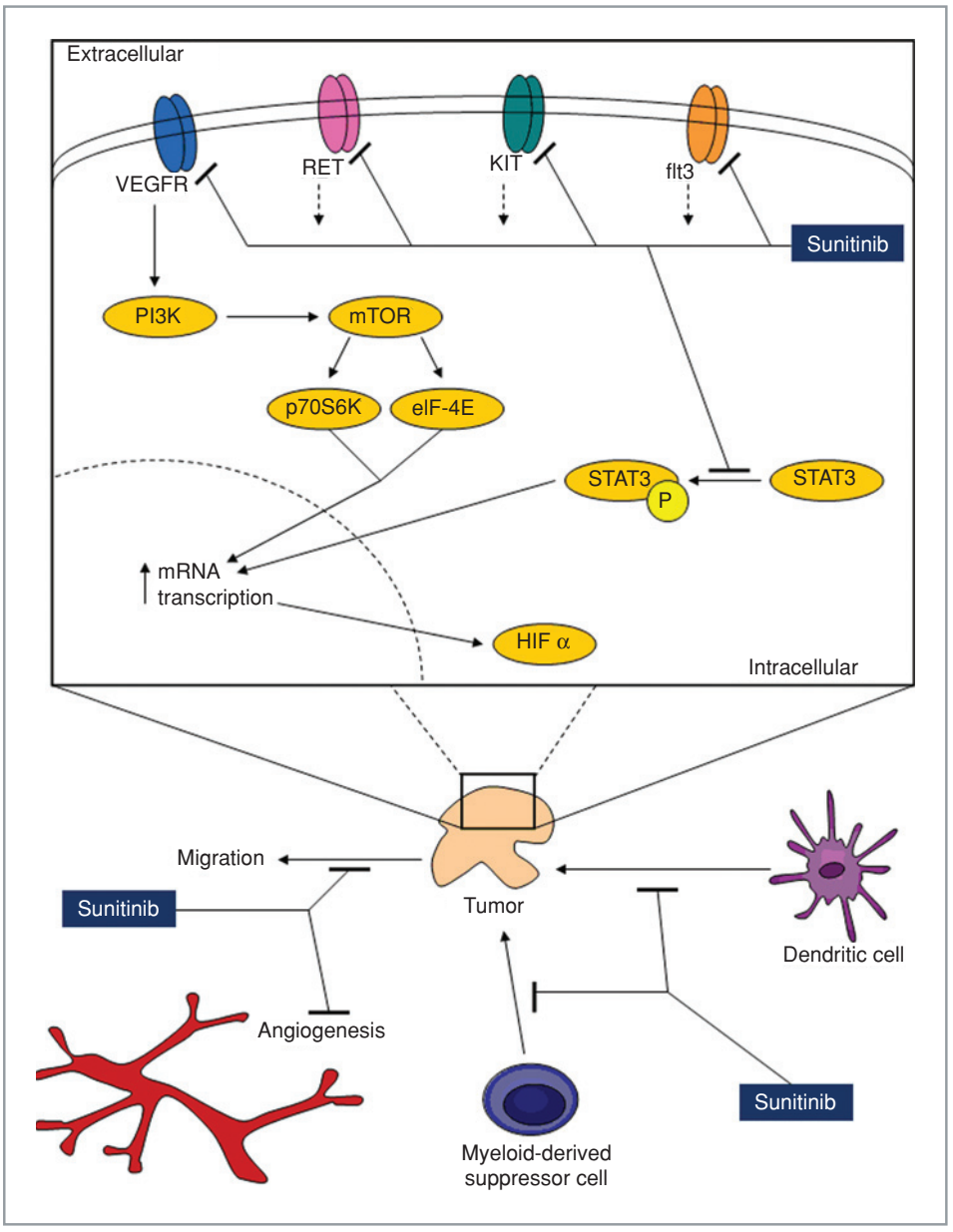

Figure 1. Agents such as sunitinib may act through a multitude of mechanisms, yielding numerous potential biomarkers of response. Adapted with permission from Pal SK, Figlin R, Yu H. Deciphering the anticancer mechanisms of sunitinib. Cancer Biol Ther 2010;10(7):712–714.

Amassing data for targeted therapies has also led to several potential clinical biomarkers of response. Perhaps most intriguingly, hypertension appears to be strongly correlated with clinical outcome in the context of several VEGF-directed agents. Using pooled efficacy data for 544 and safety data for 4,541 patients treated with sunitinib, Kaplan–Meier methods were employed to compare survival in patients with and without hypertension (71). In this study, blood pressure measurements were mandated on days 1 and 28 of each 42-day cycle. Distinguishing groups of patients based on the maximally achieved blood pressure, those patients who achieved a maximum systolic blood pressure of 140 mmHg or more had a profound improvement in both PFS (12.5 vs. 2.5 months; $P < 0.0001$) and OS (30.5 vs. 7.8 months; $P < 0.0001$), as compared to patients who achieved a maximum systolic blood pressure of less than 140 mmHg. A similar, albeit more subtle, difference in PFS and OS was observed using a diastolic blood pressure threshold of 90 mmHg. Paralleling the data for sunitinib, Cancer and Leukemia Group B trial 90206 suggests the role of hypertension as a predictor of bevacizumab activity (6, 72). This study compared bevacizumab with IFN-α to IFN-α alone in patients with treatment-naive mRCC. Patients treated with bevacizumab who incurred a grade 2 or more hypertension had both an improved PFS (13.2 vs. 8.0 months; $P < 0.001$) and OS (41.6

vs. 16.2 months; $P < 0.001$). These data are akin to that for use of bevacizumab in advanced breast cancer (73).

Complicating the use of hypertension as a predictive tool is the *post hoc* nature of this biomarker. To this end, VEGF and VEGFR SNPs that predict the occurrence of hypertension have been proposed (74). In a cohort of 64 patients with clear cell mRCC receiving sunitinib, several candidate VEGF and VEGFR SNPs were assessed. Notably, VEGF SNP 2578 was associated with a higher frequency of sunitinib-induced hypertension (C/C: 100%, A/C: 89%, A/A: 69%, $P = 0.03$). Another manner in which to incorporate biomarkers, such as hypertension, is through dose titration. A trial of the novel VEGF-TKI axitinib is employing a schema that allows for modification of dosing to elicit hypertension (75). With these studies underway, attention should also be devoted to understanding the mechanism of hypertension in this setting.

Outside of hypertension, several other clinical predictors of VEGF-directed therapy have been proposed. Most recently, Choueiri et al. presented a comprehensive analysis of 475 patients treated at US and Canadian centers with first-line VEGF-directed therapy (76). In this cohort, the impact of body–mass index (BMI) and body surface area on survival was assessed. Interestingly, obese patients (BMI > 30 kg/m^2) had a superior median OS as compared to nonobese patients (BMI $\leq$ 30 kg/m^2; 32.5 vs. 20.6 months; $P = 0.0001$). Importantly, these differences persisted even after multivariate analysis accounting for adapted MSKCC prognostic grouping.

Examining the VEGF-Signaling Axis

Moieties along the VEGF-signaling axis have been examined in detail to determine any correlation with the activity of VEGF-directed agents and mTOR inhibitors. As described previously, VEGF SNPs have been correlated with the frequency of hypertension; the same studies seem to suggest that SNPs in VEGFR2 may predict clinical outcome (74). Specifically, VEGFR SNP genotypes 889 (G/A) and 1416 (A/A) had significantly improved OS as compared with other genotypes ($P = 0.03$). DePrimo et al. have characterized circulating levels of VEGF, VEGFR2, and VEGFR3 in 63 patients with mRCC receiving sunitinib after failure of prior cytokine therapy (77). Notably, larger changes in each of these moieties were observed in patients who achieved an objective tumor response as compared to patients who exhibited stable disease or progression ($P < 0.05$ for each). In a similar fashion, Rini et al. assessed patients receiving sunitinib for bevacizumab refractory mRCC (78). In a

Table 1. Studies examining potential molecular prognostic markers in RCC

Marker	References	Description
TIMP-3	Pena et al. (81)	In the phase III TARGET trial comparing sorafenib to placebo in largely cytokine-refractory patients, baseline blood was assessed for VEGF, sVEGFR-2, CAIX, TIMP-1, Ras p21, and *VHL* mutation. In a multivariate analysis that included these biomarkers and MSKCC risk criteria, only TIMP-1 remained prognostic for OS ($P = 0.002$).
S100A4	Bandiera et al. (98)	In a series of 32 primary tumor specimens derived from patients with RCC, 5-year survival was lower in patients with high S100A4 expression as compared to weak expression (41% vs. 78%; $P < 0.05$).
IMP3	Hoffman et al. (82)	In a series of 716 primary tumor specimens derived from patients with unilateral ccRCC, IMP3 expression was associated with advanced stage and grade, as well as sarcomatoid differentiation. Positive IMP3 expression was associated with a significant increase in the risk of distant metastasis (HR 4.71; $P < 0.001$) in patients with localized disease. On multivariate analysis, IMP3 expression was associated with a 42% increase in the risk of death from RCC ($P = 0.024$).
MMP-9	Kawata et al. (99)	In a series of 120 primary tumor specimens derived from patients with localized RCC, MMP-9 was associated with high-nuclear grade (P-0.017). MMP-9 was also an independent prognostic factor ($P = 0.003$).
PI3K	Merseburger et al. (100)	In a series of 176 primary tumor specimens derived from patients with RCC, increased PI3K expression was associated with reduced survival ($P = 0.030$).
Thrombospondin-1	Zuback et al. (101)	In 172 consecutive patients with ccRCC who received nephrectomy, thrombospondin-1 expression was associated with high nuclear grade (P,0.001), advanced stage ($P < 0.001$), and tumor progression ($P = 0.006$).
Circulating tumor cells (CTCs)	Bluemke et al. (102)	In blood samples derived from 154 patients with RCC, CTCs were found in 81 specimens (53%). Detection of CTCs was correlated with lymph node status ($P < 0.0001$), presence of synchronous metastases at the time of resection ($P = 0.014$), and poor overall survival (RR 2.3; $P = 0.048$).

study of 62 patients receiving sunitinib on a standard schedule, VEGF-C and sVEGFR-3 levels decreased during the course of therapy, and lower baseline levels were associated with higher response rates and a longer PFS. A similar study by Porta et al. points to the potential predictive role of the cumulative baseline VEGF titer (79). In a study including 85 patients treated with sunitinib therapy, an above-threshold VEGF titer was associated with a median PFS of 4.7 months (95%CI: 2.8–8.3), whereas a below-threshold titer was associated with a median PFS of 11.2 months (95%CI: 6.5–15).

Downstream of VEGFR, the serine–threonine kinase p70S6K and pAKT have been proposed as potential predictors of mTOR activity in RCC (80). It is important to recognize that outside of the VEGF-signaling axis, multiple other moieties have been suggested as either potential prognostic factors (Table 1) or predictive factors for currently existing therapies (Table 2). Although the majority constitutes relatively small, hypothesis-generating efforts, several are derived from some of the largest randomized trials in RCC to date. For instance, correlative studies associated with the phase III study comparing sorafenib to placebo ($n = 903$) have identified TIMP-3 (tissue inhibitor of metalloproteinase) as an independent prognostic factor in RCC, correlating with survival even after adjustment for VEGF axis moieties and MSKCC risk criteria (81). In a similarly sized cohort of mRCC patients ($n = 716$), the insulin-like growth factor-II, mRNA binding protein IMP-3, has been associated (on multivariate analysis) with the 5-year risk of distant metastasis and OS (82). Finally, studies accompanying the randomized discontinuation trial of pazopanib have identified several potential markers of drug activity and further outline a strategy for further biomarker assessment (21). Using a platform assessing various cytokines and angiogenic factors, Heymach et al. have identified a potential association between lower baseline levels of HGF, IL-6, and IL-8 and increased tumor shrinkage in patients treated with pazopanib. It would be of interest to see whether a similar platform could be used to predict the activity of other VEGF-TKIs, as well.

Conclusions

The numerous candidate biomarkers identified herein suggest numerous potential strategies to optimize patient selection for specific targeted therapies. Validation and implementation, however, represent a major obstacle. Sargent et al. have proposed several potential trial designs for biomarker validation (83). Each requires an immense allocation of patient resources. For example, consider a marker-based strategy design (Fig. 2) employed to assess a biomarker with 50% prevalence and an ability to predict a 20% benefit from a specified agent. Assuming an acceptable power of 80% and type I error rate of 5%, approximately 2,700 patients would need to be randomized. This immense allocation of patient

Table 2. Studies describing potential predictive markers associated with a response to currently available targeted therapies

Marker	Drug	References	Description
HGF, IL-6, IL-8 (Panel)	Pazopanib	Heymach et al. (21, 103)	In 129 blood samples derived from patients enrolled in a randomized discontinuation study of pazopanib for mRCC ($n = 217$), elevated HGF, IL-8, and IL-6 at baseline correlated with less tumor shrinkage.
CAIX	Sorafenib	Choueiri et al. (104)	Tumor tissue derived from 118 mRCC patients initiating VEGF-directed therapy was assessed for CAIX expression. For patients receiving sorafenib, mean shrinkage with high CAIX was -13% vs. 9% with low CAIX ($P = 0.05$ for interaction).
TNF-α, MMP-9	Sunitinib	Perez-Garcia et al. (105)	In serum from 31 patients treated with sunitinib for mRCC, 174 cytokines were assessed. TNF-α and MMP-9 baseline levels were significantly associated with OS and time to progression ($P < 0.05$).
NGAL	Sunitinib	Porta et al. (79)	In 85 patients receiving sunitinib for mRCC, VEGF and NGAL were significant predictors of OS on multivariate analysis. The RR for NGAL was 1.91 (95%CI 1.39–2.42).
bFGF	Sunitinib	Tsimafeyeu et al. (106)	In 38 patients receiving sunitinib for mRCC, levels of basic fibroblast growth factor (bFGF) significantly increased with disease progression ($P = 0.001$). A nonsignificant decline in bFGF accompanied tumor stabilization or response.
LDH	Temsirolimus	Armstrong et al. (107)	In the pivotal phase III evaluation of temsirolimus ($n = 404$), survival was significantly improved in 140 patients who received temsirolimus and had an elevated LDH ($P < 0.002$). In 264 patients with a normal lactate dehydrogenase (LDH), survival was not improved with temsirolimus as compared to IFN-α ($P = 0.514$).

Abbreviations: RR, relative risk; NGAL, neutrophil gelatinase-associated lipocalin

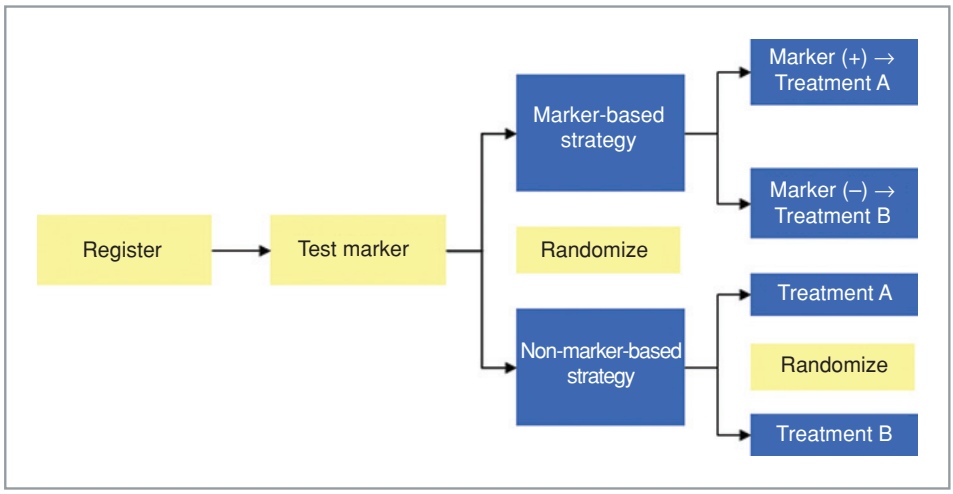

Figure 2. A proposed marker-based strategy design to evaluate predictive biomarkers. Adapted with permission from Sargent et al. (83).

resources would have to be resolved with other demands of the research community. For instance, an ever-growing pipeline of agents awaits clinical evaluation. These agents include distinct VEGF-TKIs (i.e., axitinib, tivozanib, and linifanib), as well as agents targeting novel signal transduction mediators such as fibroblast growth factor (dovitinib), Akt (MK-2206), and PI3K (BEZ235; refs. 84–90). With each agent warranting clinical trials in hundreds of patients prior to clinical implementation, the research community will be forced to decide between either assessing novel drugs or optimizing use of existing agents through detailed biomarker analyses.

In the interim, novel trial designs can be used to foster the identification of potential biomarkers. Several neoadjuvant studies have recently been reported, which provide an ideal mechanism for target validation and biomarker assessment in association with targeted therapies. For instance, a neoadjuvant trial of sorafenib in RCC included 17 patients with localized disease and 13 patients with metastatic disease (91). Radiographic correlates showed a decrease in the size of the primary tumor with loss of intratumoral enhancement; other biological correlates are pending. Bex et al. have reported correlative data from patients receiving preoperative monotherapy with either sunitinib ($n = 15$) or bevacizumab ($n = 18$; ref. 92). Correlative data from these efforts suggest alterations in the $CD4^+CD25^+Foxp3^+$ cellular population (T_{regs}), and further suggest variations in the $CD8^+/Foxp3^+$ ratio (both assessed by immunohistochemical methods in tumor tissue). As noted previously, modulation of the former population can modulate the adaptive immune system and enhance the antitumor immune response (93, 94). As these studies show, neoadjuvant and preoperative studies are safe and feasible and offer a prime opportunity for "proof-of-principle'' of preclinical observations.

Efforts in other malignancies can offer insight into potential approaches for efficient biomarker-based studies in RCC. A prime example is the Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination trial. In this study, patients are randomized to one of several agents (erlotinib/bexarotene, sorafenib, vandetanib or erlotinib) with consideration of relevant targets/predictors (such as *EGFR* mutation, *BRAF* mutation, *KRAS* mutation, etc.; ref. 95). Looking ahead, a panel of salient biological alterations in RCC could be used to direct the design of upcoming clinical trials. At present, it is critical that biomarker studies be incorporated into the comparative trials that are ongoing. For example, RECORD-3 is a randomized, phase II switching study that will juxtapose sunitinib against everolimus as first-line therapy for mRCC (96). A separate randomized, phase II study comparing sorafenib to temsirolimus as second-line therapy is ongoing (97). Aside from the obvious clinical merits of these studies, both offer a prime opportunity to identify biomarkers that may predict a superior response to either mTOR inhibitors or VEGF-TKIs. With a limited allocation of patient resources and multiple clinical and preclinical hypotheses to validate, creativity in clinical trial design is of prime importance in the research community.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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