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Target Audience

This activity is intended for urologists, oncologists, and other physicians who care for patients with RCC.

Goal

The goal of this activity is to evaluate the use of molecular pathology in the care of patients with RCC.

Learning Objectives

Upon completion of this activity, participants will be able to:

1. Distinguish the epidemiology and prognosis of RCC
2. Analyze the molecular pathology of RCC
3. Identify specific biomarkers related to RCC
4. Evaluate challenges in the search for clinically relevant biomarkers for RCC

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Author(s)

Grant D. Stewart, BSc(Hons), MBChB, MRCS(Ed), PhD

Lecturer in Urological Surgery, University of Edinburgh, United Kingdom

Disclosure: Grant D. Stewart, BSc(Hons), MBChB, MRCS(Ed), PhD, has disclosed the following relevant financial

relationships:
Received honoraria from: Pfizer Inc.
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Fiach C. O'Mahony, BSc, PhD

Edinburgh Urological Cancer Group, Western General Hospital, Edinburgh, United Kingdom

Disclosure: Fiach C. O'Mahony, BSc, PhD, has disclosed no relevant financial relationships.

Thomas Powles, MBBS, MD, MRCP

Senior Lecturer in Urology Cancer; Honorary Consultant in Medical Oncology, St Bartholomew's Hospital; UCLH, London, United Kingdom

Disclosure: Thomas Powles, MBBS, MD, MRCP, has disclosed the following relevant financial relationships:
Received grants for research support from: AstraZeneca Pharmaceuticals LP; GlaxoSmithKline; Pfizer Inc. Received honoraria from: Pfizer Inc.
Served as a speaker or on the speakers bureau for: Pfizer Inc.

Antony C. P. Riddick, MD

Consultant Urological Surgeon, Western General Hospital and St John's Hospital, Edinburgh, United Kingdom

Disclosure: Antony C. P. Riddick, MD, has disclosed no relevant financial relationships.

David J. Harrison, BSc(Hons), Ed, MD, FRCPath, FRCP(Ed)

Professor of Pathology, University of Edinburgh; Director of Laboratory Medicine, Lothian University Hospitals Division, Edinburgh, United Kingdom

Disclosure: David J. Harrison, BSc(Hons), Ed, MD, FRCPath, FRCP(Ed), has disclosed no relevant financial relationships.

Dana Faratian, MD

Senior Clinical Lecturer, University of Edinburgh, United Kingdom

Disclosure: Dana Faratian, MD, has disclosed no relevant financial relationships.

Editor(s)

Suzanne Farley

Chief Editor, *Nature Reviews Urology*

Disclosure: Suzanne Farley has disclosed no relevant financial relationships.

CME Author(s)

Charles P. Vega, MD

Associate Professor, Residency Director, Department of Family Medicine, University of California, Irvine

Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.

CME Reviewer(s)

Nafeez Zawahir, MD

CME Clinical Director, Medscape, LLC

Disclosure: Nafeez Zawahir, MD, has disclosed no relevant financial relationships.

Sarah Fleischman

CME Program Manager, Medscape, LLC

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From Nature Reviews Urology

What Can Molecular Pathology Contribute to the Management of Renal Cell Carcinoma?

Grant D. Stewart, BSc(Hons), MBChB, MRCS(Ed), PhD; Fiach C. O'Mahony, BSc, PhD; Thomas Powles, MBBS, MD, MRCP; Antony C. P. Riddick, MD; David J. Harrison, BSc(Hons), Ed, MD, FRCPath, FRCP(Ed); Dana Faratian, MD

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Abstract and Introduction

Abstract

The incidence of renal cell carcinoma (RCC) is increasing and outcomes remain poor. One-third of patients with localized disease will relapse, and 5-year survival for patients with metastatic disease is less than 10%. No molecular test is currently available to identify which patients who have undergone 'curative' surgery will relapse, and which patients will respond to targeted therapy. Some well characterized biochemical pathways, such as those associated with von Hippel-Lindau disease, are aberrantly regulated in RCC and are associated with histological subtype, but the understanding of these pathways contributes little to the clinical management of patients with RCC. Gene expression and sequencing studies have increased our understanding of the genetic basis of the disease but have failed to establish any unified classification to improve molecular stratification or to predict which patients are likely to relapse or respond to targeted therapy. Instead, they have served to highlight that RCC is heterogeneous at histological, morphological, and molecular levels, and that novel approaches are required to resolve the complexity of RCC prognostication and prediction of treatment response.

Introduction

Renal cell carcinoma (RCC) has the highest mortality rate of all the urological malignancies[1] and is the eighth most common cancer in the UK.[2] In 2007, 8,228 people in the UK were diagnosed with RCC, and there were 3,848 RCC deaths in 2008.[2] The estimated numbers of new RCC cases and deaths in the USA for 2010 are 58,240 and 13,040, respectively.[1] The reported incidence of RCC is increasing at a rate of 2.5% per year,[3] and since the 1970s RCC incidence in the UK has doubled for men and increased by 130% for women,[2] primarily owing to the use of improved imaging techniques, often resulting in incidental identification of a tumor.[4-7]

In other solid tumors, such as breast cancer, molecular pathology tests are available with which to stratify patients into good-prognosis and poor-prognosis groups through the use of characteristics beyond the standard clinical and pathological parameters, and, therefore, to identify which patients are likely to benefit from adjuvant therapy. In RCC, histopathology

remains the gold standard for determining prognosis. Patients with small, organ-confined tumors (pathological stage T1; pT1) have a 5-year survival rate >90% in contrast to ~10% in patients with distant metastasis.[2] For patients with localized tumors, total or partial nephrectomy (for patients with tumors ≤4 cm)[8] is the optimal primary treatment for RCC.[9] However, RCC will recur in 20-40% of patients after resection, depending on the stage and grade of tumor;[10] 7% of pT1 tumors recur, compared to 26% and 39% for pT2 and pT3 tumors, respectively.[11] Recurrence occurs in 13% of patients with grade 2 tumors, compared with 38% of patients with grade 3 and 38% of patients with grade 4 disease.[12] The unpredictable postoperative course, and the cost and morbidity associated with diagnostic imaging and biopsy make the follow-up of patients with localized RCC difficult. As such, there is currently no uniform agreement on the optimal follow-up regimen.[13] Although there is a trend for minimally invasive treatment (such as cryotherapy and radiofrequency ablation) or surveillance of small renal lesions, even small RCCs have the capacity to metastasize, making identification of markers for the aggressiveness of individual tumors essential.[14]

Unfortunately, metastatic RCC is uniquely resistant to chemotherapy (response rate ~6%)[15] and radiotherapy, so treatment for metastatic RCC has centered around the use of immunotherapy with interleukin 2 (IL-2) and interferon a (IFN-α).[16] Immunotherapy, however, is associated with a substantial number of adverse effects—such as fever, nausea, vomiting, diarrhea, neutropenia, fatigue, anemia, blood pressure changes and depression—and has limited efficacy. The response rate for IFN-a therapy is only 10-20%,[17] and high-dose IL-2 provides a durable response in 28% of patients (range 6.1-34.1 months), with only 6% responding completely.[18]

In view of these limited options, poor outcomes, and narrow therapeutic window, there has been a drive to use targeted therapy to improve patient outcomes in RCC—but clinical results still look less than optimal. 40% of patients receiving sunitinib will respond to the agent, with an approximate doubling of the progression-free survival compared to immunotherapy-treated controls,[17] but the vast majority of patients still relapse within 1 year.[19] A large proportion of patients have an intrinsic, genetic resistance to targeted therapies, and the vast majority of patients who do not will go on to acquire resistance to these treatments.[20] However, studies of targeted therapies in breast, colorectal, and other cancers suggest that tissue biomarker tests can specifically identify patients who are likely to benefit from therapy. For instance, in breast cancer, detection of HER2 receptor tyrosine kinase overexpression (the target of monoclonal antibody therapies such as trastuzumab) selects patients who are likely to benefit from this class of drug.[21] Leading on from this, a deep understanding of the molecular biology of cancer has resulted in rational drug design and the exploitation of cellular signaling in order to enhance treatment efficacy. Specifically, breast and ovarian tumors that harbor mutations in the DNA-repair proteins BRCA1 and BRCA2 are exquisitely sensitive to poly(ADP-ribose) polymerase (PARP) inhibition, owing to a phenomenon known as 'synthetic lethality'[22] (in which a combination of mutations in two or more genes leads to cell death), and gastrointestinal stromal tumors (GISTs) with activating mutations in the tyrosine kinase c-kit are rendered extremely sensitive to imatinib.[23]

Why is it that prognostic and predictive tests for RCC remain elusive, when similar pathology-based and molecular biology approaches have led to clinically useful tests in other diseases? In this Review, we will discuss what is known about the histopathology and molecular biology of RCC. We will review the morphological and molecular heterogeneity, pattern of spread and metastasis and hypoxic regulation of signaling in RCC, and highlight priority areas for translational research. We will also discuss some biomarkers that are promising candidates for personalizing RCC therapy. Finally, we will explore some of the hurdles unique to RCC that have hampered effective translation of these biomarkers into clinical practice, and have challenged our answering of the two fundamental clinical questions: who will relapse, and who will respond?

Histopathology and Molecular Biology

There are five main histological subtypes of sporadic RCC: clear cell (70-80%), papillary (10-15%), chromophobe (3-5%), collecting duct (1%) and unclassified (1%).[24] Although clear cell RCC (ccRCC) has the fastest rate of extrarenal growth, metastasis (most commonly to the lung, liver, bone or brain) and mortality, patterns of metastasis vary among subtypes, with papillary RCC more likely to spread to the lymph nodes and chromophobe RCC more likely to metastasize to the liver than ccRCC.[25] Of note, oncocytomas—benign tumors that often cannot be differentiated from malignant RCC by clinical or radiological means—are commonly included in RCC biomarker studies, as numerous nephrectomies for presumed RCC are subsequently diagnosed as oncocytoma on histopathology assessment.

Approximately 4% of all RCCs are hereditary.[26] Ten individual hereditary RCC conditions have been identified to date,[27] including ccRCC, type 1 and type 2 papillary RCC, chromophobe RCC, familial nonsyndromic renal carcinoma and tuberous

sclerosis complex.[28] Knowledge of the hereditary form of the disease has also improved our understanding of the pathophysiology of sporadic RCC. Similarities between sporadic and hereditary forms of RCC have been confirmed in molecular profiling studies, which showed that the two forms exhibited similar gene expression profiles and that unsupervised analysis was unable to distinguish between them.[29] The two main pathways deregulated in both hereditary and sporadic RCC and that are targets for therapy, are the von Hippel-Lindau (VHL) pathway, and that of the membrane-bound receptor tyrosine kinase, MET. Loss-of-function mutations of the *VHL* tumor-suppressor gene (located on 3p25-26), which are responsible for almost all inherited cases of ccRCC,[30] are also involved in at least two-thirds of sporadic ccRCC cases.[26] In the hypoxia pathway, loss of VHL function causes accumulation of hypoxia-inducible factor (HIF) proteins and the transcriptional upregulation of genes normally expressed under hypoxic conditions. These hypoxia-associated genes encourage cell growth and survival, and include angiogenic factors, such as vascular endothelial growth factor (VEGF) and mitogenic factors, such as transforming growth factor a (TGF-α) and platelet derived growth factor β (PDGF-β);[3] ccRCC is characteristically highly vascularized, partly due to the upregulation of angiogenesis as a result of mutations in *VHL*.[26]

Papillary RCC is the second most common subtype of inherited RCC, and is subdivided into hereditary papillary RCC (HPRCC) and hereditary leiomyomatosis RCC (HLRCC), which correspond to sporadic type 1 and type 2 papillary RCC. *HPRC*—the MET proto-oncogene that is responsible for HPRCC[3]—is located on 7q31.3, and is mutated in 5-13% of sporadic papillary type 1 RCCs.[26] MET is a receptor tyrosine kinase for hepatocyte growth factor (HGF), which, in the absence of a ligand, normally exists in an autoinhibited state.[31] Deregulation of MET results in increased mitogenesis, morphogenesis and motogenesis.[28] In addition to mutations in *MET* itself, trisomy of chromosome 7, which increases the expression of activated MET protein, occurs in HPRCC and in 75% of sporadic papillary RCC cases.[31] Several other pathways that provide potential targets for RCC treatment have been identified through study of inherited RCC, including the mammalian target of rapamycin (mTOR) pathway, growth factor signaling pathways and the Wnt/β-catenin pathway.[26,28]

Subtype Identification

Several gene expression profiling studies have attempted to take a global approach to the analysis of the molecules and pathways intrinsic to RCC and the interaction between them.[32] Although these methods were able to distinguish between normal and cancerous tissue[33-37] and identify the RCC subtypes,[33,35,37-40] they failed to establish reproducible intrinsic molecular subtypes like those shown to exist in breast cancer. The greatest discordance in RCC gene expression profiles is between ccRCC and all other RCC subtypes, with ccRCC only very rarely grouping with non-ccRCC subtypes.[41] Accurate identification of RCC subtype is essential for guiding treatment, as different subtypes have characteristic responses to therapy —ccRCC, for example, is more responsive to chemotherapy and immunotherapy than the other RCC subtypes.[42] However, without a unified classification, gene expression profiles do not add much information beyond basic histopathological subtyping.

Although histological subtyping is usually straightforward, the addition of newly described RCC subtypes— such as renal medullary carcinoma, Xp11 translocation carcinoma, carcinoma associated with neuroblastoma and mucinous, tubular and spindle cell carcinoma—is making subtyping increasingly complex.[42] Protein-based studies have attempted to identify RCC subtypes, with high levels of sensitivity and specificity.[38,43-46] Specific protein markers include carbonic anhydrase IX (CAIX) for ccRCC[47] and cytokeratin 7 (CK7) for papillary RCC;[48] however, protein marker combinations are likely to be more relevant than single proteins for identification of histological subtypes, as no single protein marker is 100% specific for a given subtype (Table 1).

Table 1.

Biomarker combination	Specificity/sensitivity for tumor type (%)	Study
Beta defensin-1, parvalbumin, and vimentin	96/96 ccRCC 57/97 pRCC 100/100 oncocytoma	Young *et al.*[43]
GST-α, AMACR, CA II, and K19	90/97 ccRCC 97/100 pRCC 80/100 chRCC + oncocytoma 97/100 TCC	Takahashi *et al.*[38]
MOC31, CD10, BerEP4, and RCCMa	93/59 ccRCC 89/51 pRCC 96/82 chRCC	Pan *et al.*[44]
Vimentin, GST-α, and EpCAM	100/100 ccRCC 100/100 chRCC 100/100 oncocytoma	Liu *et al.*[45]
AMACR, CK7 and CD10	92/86 ccRCC 97/87 pRCC 94/79 chRCC 96/78 oncocytoma	Allory *et al.*[46]

Abbreviations: AMACR, alpha-methylacyl-CoA racemase; BerEP4, mouse monoclonal antibody to EpCAM; CA II, carbonic anhydrase II; CD10, cluster of differentiation 10; CK7, cytokeratin 7; EpCAM, epithelial cell adhesion molecule; GST-α, glutathione S-transferase; K19, keratin polypeptide 19; MOC-31, monoclonal antibody which recognizes epithelial glycoprotein 2; RCCMa, renal cell carcinoma marker.

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Protein Combinations Guiding Differentiation of RCC Subtypes

Important genetic differences between normal and malignant kidney tissue include expression of genes which code for vimentin[49] and CAIX (itself a potential therapeutic target),[50] upregulation of genes involved in cell adhesion and downregulation of genes encoding transport proteins,[33,51] and high expression of genes involved in signal transduction and cellular matrix organization.[52]

Gene expression profiling has identified a number of genetic subgroups of ccRCC, with distinct survival times,[34,37,39,53-57] which could be useful for prognostic purposes. Genes identified include those involved in angiogenesis and metalloproteinase actions[34] as well as the vascular cell adhesion molecule 1 (VCAM 1) which could be used alone to stratify patient survival.[56] The potential of a tumor to metastasize has also been studied by comparing the gene expression of tissue from metastatic sites with those of aggressive primary tumors and normal tissue. This approach identified 35 genes for predicting tumor aggressiveness.[54] A similar study identified 31 genes that showed differing expression across normal, primary tumor and metastatic tumor tissue, as well as 155 genes that could be used to predict metastatic status.[35]

Expression of Potential Biomarkers

Expression of candidate biomarkers has also been assessed. RCC-specific markers are focused on ccRCC, and are usually subdivided into those associated with the VHL pathway or the mTOR pathway.[58-61]

Biomarkers From the VHL Pathway. Potential biomarkers related to the VHL pathway include *VHL* itself, HIF, VEGF, and CAIX. The use of *VHL* and HIF-1α as prognostic or predictive biomarkers is contentious, as studies of *VHL* mutation status as a prognostic[62-64] (Table 2) and predictive biomarker[65-68] (Table 3) have produced conflicting results. Similarly, HIF-1α overexpression as a predictive biomarker is also controversial, as it has been associated with both a worsened[69] and an improved prognosis.[70] The use of HIF-1α as a biomarker has also been complicated by the finding that elevated HIF-1α levels can be independent of VHL inactivation, and could be due to hypoxia-mediated mechanisms, or an artifact of surgery.[60] The other two isoforms of HIF-α (HIF-2α and HIF-3α), which are upregulated following inactivation of the *VHL* gene, have not been evaluated as biomarkers.

Table 2.

Marker	Sample size	Effect of high expression on prognosis	Study
RCC-specific			
VHL	187	Improved prognosis for ccRCC	Yao *et al.*[62]
	113	Improved prognosis for ccRCC	Schraml *et al.*[63]
	150	No effect on ccRCC prognosis	Baldewijns *et al.*[64]
HIF-1α	176	Improved prognosis for ccRCC	Lidgren *et al.*[70]
	308	Worsened prognosis for ccRCC	Klatte *et al.*[69]
VEGF	164	Worsened prognosis for RCC	Jacobsen *et al.*[71]
	33	Worsened prognosis for RCC	Na *et al.*[72]
	903	Worsened prognosis for RCC	Escudier *et al.*[73]
VEGFR	340	Improved prognosis for ccRCC	Lam *et al.*[78]
CAIX	231	Improved prognosis for ccRCC	Bui *et al.*[47]
	730	No effect on ccRCC prognosis	Leibovich *et al.*[79]
pS6	375	Worsened prognosis for RCC	Pantuck *et al.*[81]
PTEN	375	Improved prognosis for RCC	Pantuck *et al.*[81]
p-AKT	375	Improved prognosis with nuclear staining, but worsened prognosis with cytoplasmic staining in RCC	Pantuck *et al.*[81]
Generic			
KI67	206	Worsened prognosis in RCC	Delahunt *et al.*[107]
	73	Worsened prognosis in ccRCC	Rioux-Leclercq *et al.*[108]
	257	Worsened prognosis in ccRCC	Visapaa *et al.*[109]
	244	Worsened prognosis in RCC	Bui *et al.*[110]
p53	73	Worsened prognosis in ccRCC	Rioux-Leclercq *et al.*[108]
	246	Worsened prognosis in RCC	Zigeuner *et al.*[111]
B7-H1	268	Worsened prognosis in RCC	Thompson *et al.*[112]
MMP2	153	Worsened prognosis in RCC	Kallakury *et al.*[113]
MMP9	153	Worsened prognosis in RCC	Kallakury *et al.*[113]
TIMP1	153	Worsened prognosis in RCC	Kallakury *et al.*[113]
TIMP2	153	Worsened prognosis in RCC	Kallakury *et al.*[113]

Abbreviations: B7-H1, B7 homolog 1; CAIX, carbonic anhydrase 9; HIF-1α, hypoxia-inducible factor 1, alpha subunit; KI67, antigen Ki67; MMP, matrix metalloproteinase; p53, protein 53; p-AKT, phosphorylated Akt; pS6, phosphorylated S6 ribosomal protein; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TIMP, tissue inhibitor of metalloproteinases; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; *VHL*, von Hippel Lindau gene.

RCC-specific and Generic Cancer Prognostic Biomarkers

Table 3.

Marker	Sample size	Tumor type	Effect of high expression on treatment response	Study
RCC-specific				
VHL	56	ccRCC	No effect on response to IL-2	Kim *et al.*[68]
	43	ccRCC	Improved response to interferon α with bevacizumab, sunitinib, or axitinib	Rini *et al.*[67]
	20	RCC	No effect on response to temsirolimus	Cho *et al.*[66]
	123	RCC	No effect on response to VEGF-targeted therapies	Choueiri *et al.*[65]
VEGF	61	RCC	Worsened response to sunitinib	Rini *et al.*[74]
	564	ccRCC	No effect on response to interferon plus bevacizumab	Escudier *et al.*[77]
	60	RCC	Worsened response to IL-2	Sabatino *et al.*[76]
	903	RCC	No effect on response to sorafenib	Escudier *et al.*[73]
	85	RCC	Worsened response to sunitinib	Porta *et al.*[75]
CAIX	58	ccRCC	Improved response to IL-2	Atkins *et al.*[80]
pS6	20	RCC	Improved response to temsirolimus	Cho *et al.*[66]
pAKT	19	RCC	Improved response to temsirolimus	Cho *et al.*[66]
Generic				
NGAL	85	RCC	Worsened response to sunitinib	Porta *et al.*[75]
IL-8	20	ccRCC	Worsened response to sunitinib	Huang *et al.*[114]

Abbreviations: CAIX, carbonic anhydrase 9; ccRCC, clear cell renal cell carcinoma; IL-2, interleukin 2; IL-8, interleukin 8; NGAL, neutrophil gelatinase-associated lipocalin; pAKT phosphorylated Akt, pS6, phosphorylated S6 ribosomal protein; RCC, renal cell carcinoma; VEGF, vascular endothelial growth factor; *VHL*, von Hippel Lindau gene.

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RCC-specific and Generic Cancer Biomarkers and Prediction of Treatment Response

The downstream VHL-pathway-associated biomarkers VEGF and CAIX are more promising. The VEGF family has been shown to be a promising prognostic and predictive biomarker for RCC. Specifically, reduced baseline levels of VEGF are associated with longer progression-free survival in numerous studies.[71-73] Studies investigating VEGF as a predictive biomarker showed that decreased levels of VEGF were associated with a positive response to sunitinib[74,75] and IL-2[76] treatment, but not with response to sorafenib[73] or IFN-α2a plus bevacizumab.[77] In contrast to VEGF itself, low expression of VEGF receptor 3 (VEGFR-3) is associated with worsened disease-free survival.[78] As a predictive marker, a low baseline VEGFR-3 level was associated with a positive response to sunitinib treatment.[74] High levels of CAIX have also been associated with improved prognosis; however, the value of CAIX as an independent indicator of survival has been debated, as other studies have shown that low CAIX levels are not associated with survival when adjusted for nuclear grade or coagulative tumor necrosis.[47,79] Patients with high CAIX levels have an improved response to IL-2,[80] illustrating the usefulness of CAIX as a predictive biomarker, and highlighting the ability of biomarkers to select for patients that will respond favorably to a particular therapy.[18]

Biomarkers From the mTOR Pathway. Potential biomarkers identified in the mTOR pathway include phosphorylated S6 ribosomal protein (pS6), elevated levels of which are associated with increased tumor grade and stage, metastasis[81] and response to temsirolimus.[66] Other mTOR biomarkers include PTEN (which is associated with lower T classification and localized disease), and the nuclear and cytoplasmic expressions of p-AKT—both of which are highly relevant in both localized and metastatic RCC (Table 2). High expression of p-AKT has also been associated with an improved response to temsirolimus therapy (Table 3).[66]

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NGAL	85	RCC	Worsened response to sunitinib	Porta *et al.*[75]
IL-8	20	ccRCC	Worsened response to sunitinib	Huang *et al.*[114]

Abbreviations: CAIX, carbonic anhydrase 9; ccRCC, clear cell renal cell carcinoma; IL-2, interleukin 2; IL-8, interleukin 8; NGAL, neutrophil gelatinase-associated lipocalin; pAKT phosphorylated Akt, pS6, phosphorylated S6 ribosomal protein; RCC, renal cell carcinoma; VEGF, vascular endothelial growth factor; *VHL*, von Hippel Lindau gene.

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RCC-specific and Generic Cancer Biomarkers and Prediction of Treatment Response

In addition to the use of single biomarkers, a number of studies have utilized the prognostic power of multiple biomarkers in combination with clinical and pathological factors, with promising concordance index values.[82,83] A study by Kim and colleagues,[83] which investigated the biomarkers p53, CAIX, gelosin and vimentin in addition to metastatic status, T stage and Eastern Cooperative Oncology Group (ECOG) performance status, produced a concordance index of 0.79. However, Klatte *et al*.[82] achieved a concordance index value of 0.904 when KI67, p53, endothelial VEGFR-1, epithelial VEGFR-1, and epithelial VEGF-D were used in combination with T classification and ECOG performance status.[82]

Lack of Clinical Impact

It is clear that our substantial understanding of the molecular basis of RCC, and considerable effort in identifying prognostic and predictive biomarkers, have not contributed to current clinical decision-making. In order to explore the reasons why this might be the case, the challenges facing translational research in RCC can be divided into methodological, biological, and pathological obstacles. Although many of these are not unique to RCC, there are some RCC-specific factors that also need to be considered.

Methodological Challenges

There are three basic qualities a robust biomarker should possess: the assay should be specific, the biomarker should be expressed homogeneously within the specimen, and finally, the marker itself should be biologically relevant. Other factors, such as adequate or appropriate controls and the manner in which the sample is collected, stored and manipulated are also important in tissue studies. Most of the biomarkers previously tested in RCC have failed on at least two of these criteria. An obvious limitation of many of the studies is statistical: most are underpowered and none conform to the REMARK (Reporting recommendations for tumor marker prognostic studies) guidelines, which have been written to enable uniform design and reporting of biomarker studies.[84] Furthermore, initial findings have often not been validated in independent cohorts or subsequent studies.

Whereas it is relatively simple to achieve specificity with DNA-based or RNA-based assays, protein assays can be more

problematic. In formalin-fixed, paraffin-embedded tissue, which constitutes the majority of archival tissue in pathological practice, proteins are crosslinked and even after the process of antigen retrieval, have a very different conformation to the short peptides against which the antibodies were raised. Assessing specificity for each assay is, therefore, paramount. In the research setting, immunohistochemistry is often poorly performed and specificity is not adequately proven. For most (non-phospho) antibodies, this would include indirect measures, such as western blotting to show a single band (with appropriate controls, preferably in overexpressing or knockdown systems), but also direct measures of specificity, including expected expression in normal control tissue, correct subcellular localization, correct tissue localization (epithelial or stromal) and, ideally, appropriate expression in overexpressing or knockdown cell lines or tissues. In our current practice, we reject 50-60% of antibodies because they fail to meet these standards.

Homogeneous expression is important, as biomarkers that show variance in expression across tumors are liable to sampling bias, particularly in small biopsy samples. As assays become more quantitative, this issue becomes even more important, as it introduces unacceptable errors into the measurement. This effect might explain some of the contradictory results in assays measuring hypoxia-related pathways, such as HIF-1α and CAIX, which show very heterogeneous expression in RCC (Figure 1) owing to intrinsic VHL-related pathway aberrations and abnormal microvasculature and necrosis, both of which are commonly seen in RCC. Morphological heterogeneity is also frequently observed, which is likely to represent even greater areas of molecular heterogeneity.

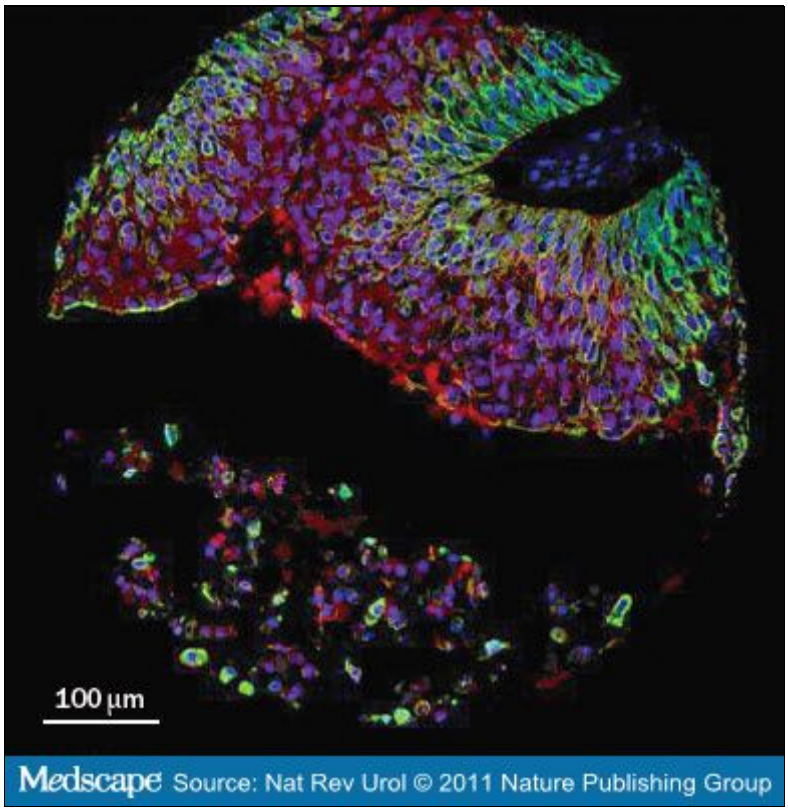

Figure 1. Intratumoral Heterogeneity of Carbonic Anhydrase IX (CAIX) Expression (Red Signal) in Renal Cell Carcinoma, by Automated Quantitative Analysis. Green signal represents tumor masking. The expression of CAIX varies greatly across this sample, despite representing a small area of a single tumor, and demonstrates the heterogeneity that can be seen in a single tumor.

Biological Challenges

Even single marker molecular tests that are considered successful, such as HER2 receptor status, have a very low positive predictive value—only 30-40% of HER2-positive patients respond to treatment with trastuzumab.[85] It is, therefore, becoming clear that the target alone is often a poor surrogate for the underlying tumor biology, particularly in providing predictive markers for targeted agents whose mechanism of action relies on the inhibition of complex signaling pathways to produce antiproliferative or proapoptotic effects. Target pathways are intrinsically robust to perturbation, owing to extensive crosstalk

and precise feedback and feedforward control. Any newly developed predictive assay will, therefore, need to be multiparametric in order to account for the complexity of the network it measures.[86]

In lung cancer, a superior response to the EGFR tyrosine kinase inhibitor erlotinib is seen in patients with mutated or increased copy numbers of *EGFR*.[87] Patients with metastatic colorectal cancer who harbor *KRAS* mutations do not benefit from treatment with the anti-EGFR monoclonal antibody cetuximab. In breast cancer, *PTEN* expression identifies women who will respond to trastuzumab.[88] However, two studies have shown that *KRAS* mutation status alone as a surrogate marker of MAPK pathway activation is insufficient for predicting the therapeutic response or resistance to anti-EGFR drugs, as KRAS-mutant cancer cells are also dependent on NFκB signaling, inhibition of which can reduce tumor growth in RAS-induced lung cancer.[89,90] These data suggest that single molecular targets are poor surrogates of pathway status. The system is so complex that prediction could be improved by better understanding, and measuring of, pathway activation and interdependency. The complexity of these pathways is illustrated in a study showing that, in mice given short-term tyrosine kinase inhibitor therapy for a primary tumor, metastatic tumor growth was actually accelerated and overall survival reduced.[91,92] Acceleration of metastasis was also observed in mice receiving sunitinib before intravenous implantation of breast cancer and skin cancer cells, suggesting possible "metastatic conditioning" in multiple organs; however, this is in contrast to the tumor growth inhibition seen when an orthotopically grown mouse tumor model was treated with sunitinib. If they can be translated to human RCC, these findings have potentially huge implications with respect to duration of therapy, mechanism-based combination therapy to prevent this double effect of monotherapy, and the role of neoadjuvant therapy.[92]

Pathological Challenges

Histological and Genetic Heterogeneity. It is quite clear that the various histological subtypes of RCC, although closely related, are very different oncological entities. Furthermore, RCCs from different patients with similar pathological grade and stage can be extremely heterogeneous, and patients with similar clinical presentations can have very different molecular profiles at a transcriptomic or genomic level.[22,93,94] Sequencing studies of RCC have demonstrated substantial genetic heterogeneity in a cancer type dominated by mutations in a single gene.[94] *VHL* mutations cause senescence of tumor cells, but a 'second hit' is required to drive ccRCC development. Sequencing of the protein-coding sequences of the genome (exome sequencing) has been used to identify other common mutations in sporadic ccRCC. *PBRM1,* a chromatin-remodelling complex gene, was found to be mutated in 41% of ccRCC cases analyzed.[95] Furthermore, genomic sequencing has demonstrated a frequent loss of histone methyltransferase genes in sporadic ccRCC, revealing a potential novel tumor suppressor gene in ccRCC.[96] These findings illustrate the heterogeneity of this disease process, whereby there is a high level of clinical and pathological variation despite supposedly common mutations. This heterogeneity might explain the different responses to therapy observed in patients with seemingly similar tumors.[22]

Morphological (intratumoral) heterogeneity is also seen in RCC, and commonly takes two forms. The first type comprises areas of dedifferentiation, known as sarcomatoid change, which are well described in RCC and confer a poor prognosis.[97] The second form consists of more subtle areas of morphological variability (Figure 2), which are more difficult to quantify by histological examination. Gene expression and genotyping studies do not take heterogeneity into account and even if microdissection methods are used, subtle phenotypic differences (which are likely to represent underlying molecular differences) can be missed.

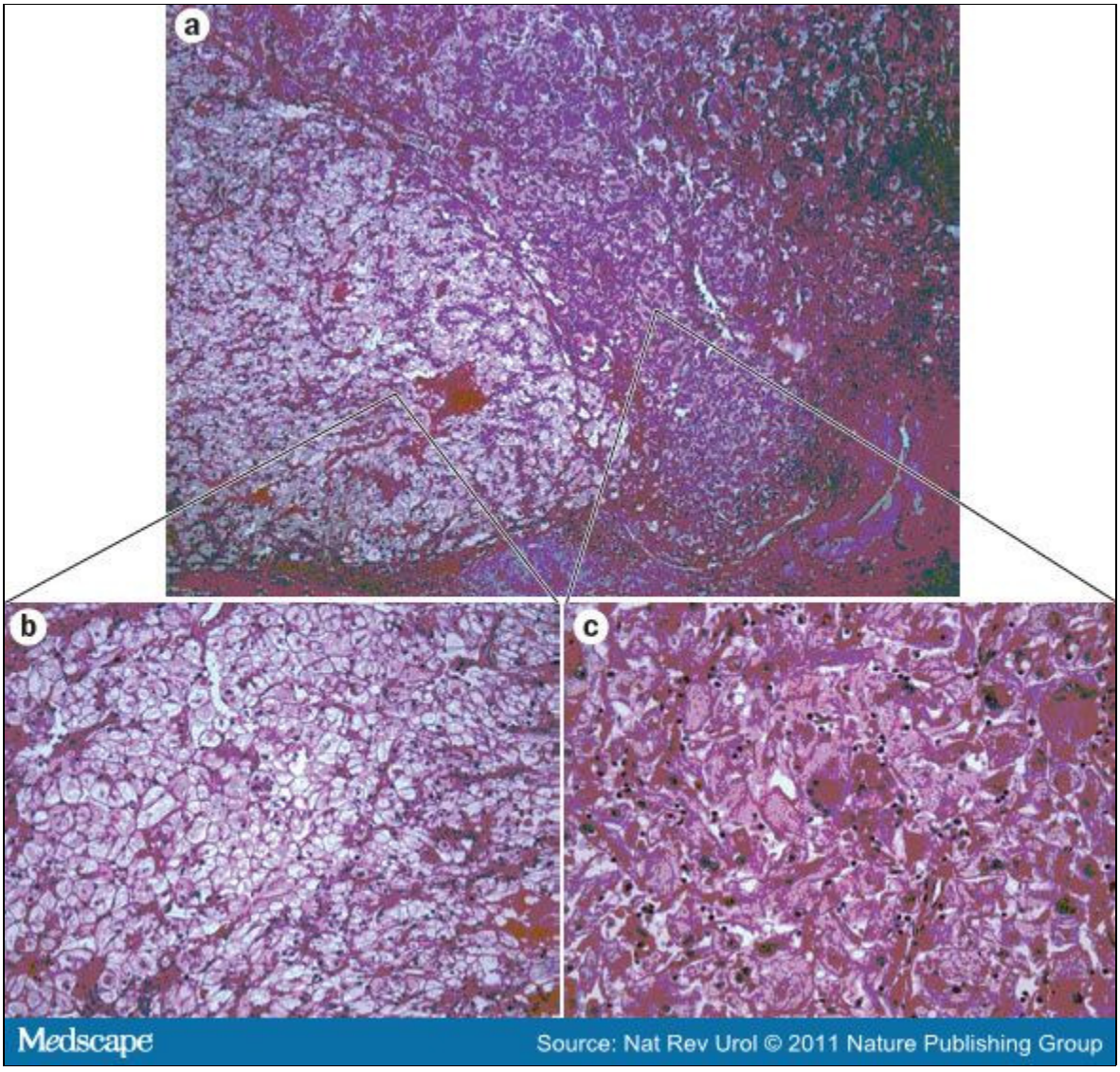

Figure 2. Hematoxylin and Eosin Staining of a ccRCC Nephrectomy Sample Illustrating Intratumoral Heterogeneity. **a** | Low power view (x100) showing two clear areas of ccRCC that are morphologically different. **b** | This area exhibits clear cell differentiation, the commonest histological subtype, with cytoplasmic clearing caused by abundant intracellular glycogen (x200). **c** | This area exhibits different morphological features, including pleomorphic muclei (x200). These two regions are adjacent to each other with minimal separation. Abbreviation: ccRCC, clear cell renal carcinoma.

Whether these subtle phenotypic changes are driven by genetic, epigenetic, transcriptional, or post-translational changes is unclear. However, without detailed analysis, robust biomarkers will prove elusive, sampling error will confound clinical studies, and, in the era of next-generation sequencing (NGS), using techniques such as exome sequencing, mutations are likely to be missed or misinterpreted. An NGS study showed that pancreatic carcinomas were highly heterogeneous at the genetic level and only certain subclones were present in meta-static disease.[98] If such heterogeneity exists in RCC, this will have profound implications for the efficacy of targeted therapy in both primary and metastatic disease. Detailed heterogeneity mapping is required in order to establish whether heterogeneity is itself an independent prognostic factor, and how it affects successful therapy.

Stromal-epithelial Interaction. The vast majority of basic cancer research is based on investigation of the epithelial cancer cell. However, there is growing evidence that the other cells in the tumor microenvironment—such as stromal cells, endothelial cells and immunological cells—have key roles in cancer development and growth. The stromal-epithelial cell interaction is especially important, and there is evidence in some cancers that the stroma controls the epithelium rather than vice versa, although there has been little research in this area in RCC specifically. In RCC, clinically relevant doses of the VEGF-targeted therapy sunitinib have been shown to act on the endothelium rather than the epithelium.[99] Furthermore, in a solid tumor such

as RCC, endothelial networks are constantly being modeled and remodeled, influencing tumoral oxygen, nutrient, glucose and pH conditions.[100] Future studies must, therefore, incorporate investigation of the nonepithelial compartments of the tumor. Endothelial cell lines should be evaluated alongside epithelial cell lines, and techniques that are able to assess the different compartments of the tumor should be used for *in situ* evaluation of protein and nucleic acid expression. Finally, the poorly understood changes that occur in a tumor following therapy will be of great importance in the decision to use synchronous or asynchronous combination therapy.[101]

The Next Generation Diagnostic Test

The design of a new diagnostic test is, therefore, challenging. The hurdles can be loosely categorized into clinical, pathological, and technical obstacles. A successful diagnostic test in RCC needs to be based around a defined clinical question, must be multiparametric (in order to address pathway complexity), and the assay needs to be robust (Figure 3).

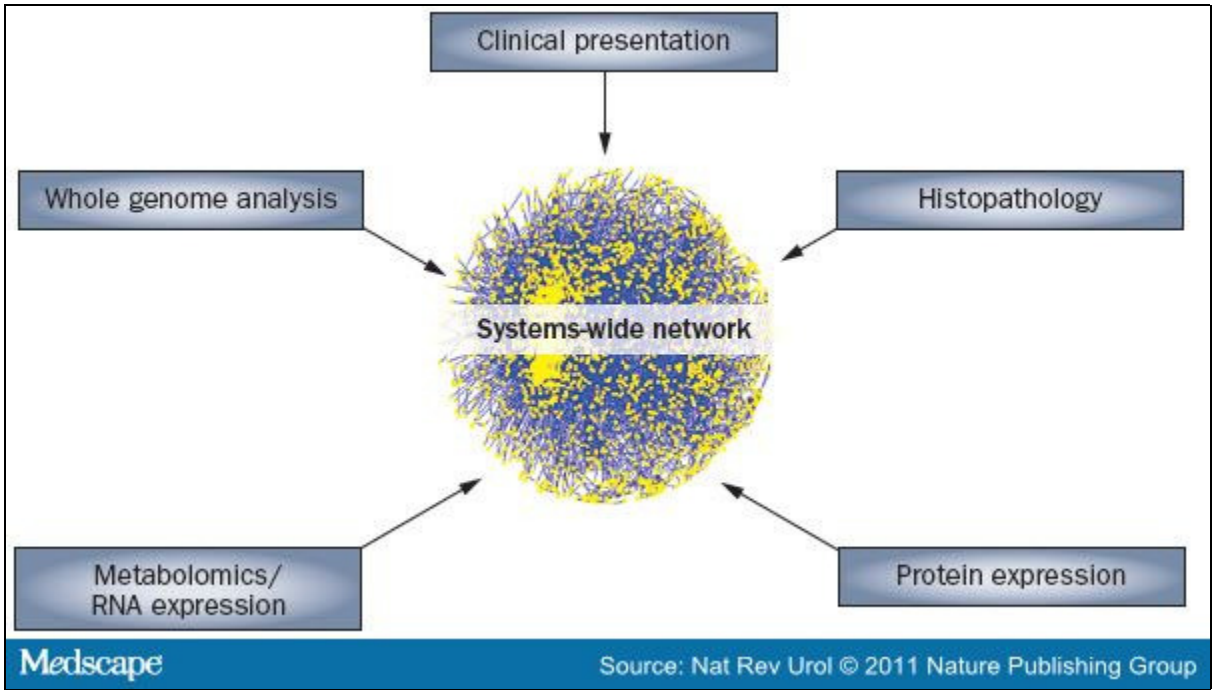

Figure 3. Development of the Next Generation of Diagnostic Test. The next diagnostic, prognostic and predictive tests will not be single proteins or clinical features but will be multifactorial, while remaining manageable. This test will be developed by resolving multilevel biological information (genetic, transcriptomic, proteomic), while retaining important clinical and traditional histolopathological information. These data will be fed into a systems biology analysis whereby techniques such as machine learning and functional interaction networks will guide development of a manageable panel of markers for the diagnostic test. Central image courtesy of Dr Ian Overton (MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Western General Hospital, Edinburgh, UK).

As many biomarker studies in RCC have been inadequately powered and RCC is a relatively rare disease, collection and storage of clinically annotated material is central to the successful design of prognostic and predictive assays. With this in mind the prospective collection of such information is essential. The Scottish Collaboration on Translational Research into RCC (SCOTRRCC) has adopted recommendations for this, and, throughout Scotland, consent is routinely sought for tissue donation and linking of patients' clinical information. Details of patient presentation, imaging, surgery, pathology and follow-up are collected for use with the linked biosample. This style of data collection also provides the added benefit of having the potential to be used for development of country-wide follow-up protocols for RCC. A further key component of the approach used in SCOTRRCC is to develop a multidisciplinary research team comprising a surgeon, oncologist, nurse, radiologist, histopathologist, molecular pathologist, cellular or molecular biologist, systems biologist, data manager, tissue banking team, IT support, charity funding, pharmaceutical company support and government input, all of whom will have a role in improving the quality of translational research.

In addition to a robust biorepository, the SCOTRRCC initiative will provide high-quality clinical material and well-annotated outcome data. In standard clinical practice, the determination of when an individual patient responds to treatment or has a disease relapse is often inaccurate. In the setting of a clinical trial, these timepoints are clearly defined and more easily determined, as serial measurement or imaging studies are more uniform and more carefully recorded than they are in practice. Fresh or fixed tumor tissue from the patients enrolled in these clinical trials is an invaluable resource for study. In the context of identifying predictive biomarkers, the development of clinical trials in which sequential tissue samples are taken before and after targeted therapy—for example, the SuMR (sunitinib in metastatic renal cancer) trial[102] and the PREDICT consortium trial[20]—are even more useful. It is not routine to perform a renal biopsy before nephrectomy for RCC, as diagnosis is usually made on imaging. As such, trials of neoadjuvant targeted therapies, with a biopsy taken before commencing drug treatment and then a subsequent nephrectomy sample, are a very exciting resource, as they encompass the period of acquired resistance of the tumor to the drug. These types of tissue sets enable us to identify molecules that are differentially expressed during the natural history of RCC. In a similar fashion, 'window of opportunity' studies enable the acquisition of tissue from patients who have been treated with a drug before their definitive surgical resection. Patients should ideally undergo a second biopsy during response and relapse phases of treatment, in order to identify pharmacodynamic markers and biomarkers for resistance.[103]

In order to address some of the technical limitations of immunohistochemistry, medium-throughput technologies for biomarker discovery and validation have been adopted. Proteins are easily measurable using routine pathology methods and, as such, are key to the development of clinically relevant tests. As most potential molecular markers and targets are proteins, proteomic profiling is expected to yield increasingly accurate answers to functional and pharmacologic questions than transcriptional profiling.[103] Reverse-phase protein arrays (RPPAs) and antibody microarrays are an improvement on western blots and enzyme-linked immunosorbent assays (ELISA) for analysis of protein lysates from fresh tumor tissue. RPPA is a relatively novel, sensitive, high-throughput technology whereby protein samples from *in vitro* and *in vivo* samples are robotically spotted onto nitrocellulose-coated glass slides and probed with a single or duplexed antibody. All samples are spotted at the same time, making RPPA ideally suited for retrospective analysis of a large number of archival clinical specimens, and addition of technical and biological replicates is simple as each spot requires only picoliters of sample.[86] RPPA enables the screening of large numbers of candidate markers and can concurrently evaluate activation, proliferation, apoptosis, or any process for which high-quality antibodies exist.[103] Using this technique, activation of TGF-β signaling was shown to be associated with chemoresistance in ovarian cancer, demonstrating successful application of proteomic technologies in biomarker development.[104]

Where the vast amount of archival formalin-fixed, paraffin-embedded tissue samples in our pathology archives is linked to patient outcome data, it is appealing to make use of them. However, to date, researchers have been reliant on semiquantitative immunohistochemistry. The development of automated quantitative analysis (AQUA), is a great advance in the field. AQUA involves *in situ* protein quantification on automated image analysis platforms for total and phosphorylated (usually active) protein levels.[105] Carefully validated antibodies can be multiplexed against proteins in any cellular compartment. We have recently applied the quantitative immunofluorescence approach to subclassifying ovarian cancers on the basis of phosphoprotein expression in order to identify new groups who might benefit from combinations of targeted therapies.[106] It is anticipated that a similar approach can be used in RCC and then validated in the neoadjuvant setting in order to optimally select therapeutic regimens.

Novel genetic techniques, such as array comparative genomic hybridization, exome sequencing and Sequenom® (Sequenom, San Diego, CA, USA), which enable deep sequencing and transcriptional analysis, are providing previously unheard of amounts of data at reasonable cost. Integration of genetic data with functional genomic techniques, such as large-scale RNA interference screening, will validate functionally important genes or transcripts.[20] Development of a next generation diagnostic test using detailed, multiscale analysis of annotated clinical material is likely to yield an assay which is multiparametric with measurements being made at all levels of systems complexity—protein, DNA, and RNA.

Conclusions

We are entering an exciting new era, in which a vast range of targeted therapies could benefit patients with RCC. So far, population-based randomized controlled trials of these agents are providing very mixed outcomes. From a pathological and molecular standpoint, RCC is a very heterogeneous malignancy. As such, it is not surprising that, if administered in an

untargeted fashion, these agents fail to evoke the responses expected. Previous marker-based studies have failed in cancer research for a number of reasons, but tumor heterogeneity amplifies these difficulties in RCC. The introduction of new protein and genetic technologies will offer new options for determining individual patient prognosis, and could help guide administration of targeted therapy and follow-up protocols in patients with RCC.

Key Points

- Despite the prominence of molecular-targeted therapy in renal cell carcinoma (RCC), and the wealth of information on pathobiology, there are no molecular pathology tests to guide disease prognosis or predict treatment response
- Both candidate and systematic approaches to selecting potential prognostic and predictive biomarkers in RCC from genetic and proteomic studies have failed
- Generic and RCC-specific methodological, biological and pathological factors can account for the failure of previous studies to identify robust biomarkers in RCC
- There is a high level of molecular and pathological heterogeneity in RCC, which has not been taken into account by existing genetic and proteomic approaches
- More-detailed heterogeneity mapping is required to establish the importance of heterogeneity in prognosis and prediction of therapeutic response
- Future predictive assays in RCC will need to be multiparametric, and incorporate novel, high-throughput, highly quantifiable technologies to account for the complexity of the pathway networks involved

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Reprint Address

Edinburgh Urological Cancer Group, Division of Pathology, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh EH4 2XU, UK. Grant D. Stewart, BSc(Hons), MBChB, MRCS(Ed), PhD grant.stewart@ ed.ac.uk

Author contributions

G. D. Stewart, F. C. O'Mahony and D. Faratian researched data for, and wrote, the article. All authors made a substantial contribution to discussions of content and were involved in the review and editing of the article before submission.

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