Tissue-Based Research in Kidney Cancer: Current Challenges and Future Directions

Sabina Signoretti,[1] Gennady Bratslavsky,[4] Frederick M. Waldman,[7] Victor E. Reuter,[8] John Haaga,[9] Maria Merino,[5] George V. Thomas,[10] Michael R. Pins,[11] Towia Libermann,[2] John Gillespie,[12] Joseph E. Tomaszewski,[13] Carolyn C. Compton,[6] Andrew Hruszkewycz,[14] W. Marston Linehan,[4] and Michael B. Atkins[3]

Abstract The past several years have seen unprecedented advances in the application of various therapeutic strategies for the treatment of patients with renal cancer. The availability of active immunotherapy, antiangiogenic therapy, and targeted therapy for this disease has brought front and center issues related to choosing the appropriate treatment for particular patient populations. It is increasingly evident that the most promising treatment selection strategies will incorporate identifying specific features of the tumor itself. To facilitate this move toward personalized medicine, it is critically important to establish some standard principles for renal cancer tissue collection, preparation, and analysis for translational research studies. In this article, we identify and discuss some critical issues related to tissue-based kidney cancer research. We focus on five major areas as follows: (*a*) surgical and image-guided techniques for tissue collection; (*b*) quality control of specimen collection, processing, storage, and review; (*c*) issues related to analysis of paraffin embedded tissues; (*d*) genomic studies; and (*e*) assessment of reproducibility of assays across institutions. In addition, some practical implementation strategies are proposed. Although many of the topics discussed are specific for renal cancer, several are also relevant to tissue based biomarker investigations in a broad array of malignancies.

In the past few years, considerable progress has been made in the treatment of kidney cancer. Novel treatments targeting molecular pathways known to be dysregulated in this disease have shown antitumor activity in the majority of patients with advanced renal cancer. The most promising treatment selection strategies will likely incorporate identifying specific features of the tumor itself (1, 2). Thus, the development of standard protocols and procedures for renal cancer tissue collection, preparation, and analysis is very critical for the advancement of the kidney cancer field. In recognition of the importance of these issues, the National Cancer Institute (NCI) supported a 1-day Workshop to discuss research aimed at discovering and validating clinically relevant biomarkers for renal cell carcinoma (RCC). The Workshop brought together pathologists, urologists, medical oncologists, and scientists working in the renal cancer biomarkers field and promoted a multidisciplinary discussion on data accuracy and reproducibility in tissue-based kidney cancer research. This article discusses the major challenges in the use of tissue specimens for translational research in kidney cancer identified in the Workshop.

Surgical and Image-Guided Techniques for Tissue Collection

Surgery. Several surgical variables are known to potentially alter mRNA and protein expression levels within the resected tissue and, consequently, bias the results of molecular analyses. As molecular studies of human tumors are increasingly used for the identification of novel diagnostic, prognostic, and predictive biomarkers, and therapeutic strategies for kidney

Authors' Affiliations: [1]Department of Pathology, Brigham and Women's Hospital and Department of Medical Oncology, Dana-Farber Cancer Institute; [2]BIDMC Genomics Center; and [3]Division of Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts; [4]Urologic Oncology Branch, Center for Cancer Research; [5]Laboratory of Pathology; and [6]Office of Biorepositories and Biospecimen Research, National Cancer Institute, Bethesda, Maryland; [7]Departments of Laboratory Medicine and Urology, Comprehensive Cancer Center, University of California San Francisco, San Francisco, California; [8]Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York; [9]Department of Radiology, University Hospitals of Cleveland and Case Western Reserve University School of Medicine, Cleveland, Ohio; [10]Department of Pathology, University of California Los Angeles, Los Angeles, California; [11]Department of Pathology, Advocate Lutheran General Hospital, Park Ridge, Illinois; [12]Science Applications International Corporation-Frederick, Inc., National Cancer Institute at Frederick, Frederick, Maryland; [13]Division of Cancer Treatment and Diagnosis, National Cancer Institute; and [14]Organ Systems Branch, National Cancer Institute, Rockville, Maryland

Received 10/25/07; revised 1/9/08; accepted 1/21/08.

Grant support: Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research and in part by a supplemental grant to the Dana-Farber/Harvard Cancer Center Kidney Cancer Specialized Program of Research Excellence. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

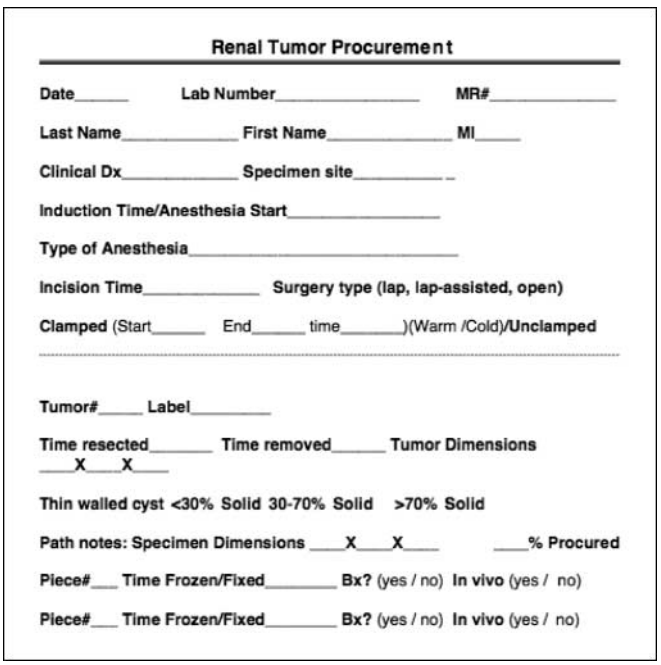
Requests for reprints: Sabina Signoretti, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115. Phone: 617-525-7437; Fax: 617-264-5169; E-mail: ssignoretti@partners.org.

doi:10.1158/1078-0432.CCR-07-4733

cancer, there is a pressing need to standardize tissue collection procedures. Four main surgical variables that may exert a significant effect on tissue genomics and proteomics can be identified as follows: (*a*) type and duration of anesthesia; (*b*) presence and duration of tissue ischemia after renal artery clamping; (*c*) size of tissue sample; and (*d*) tissue storage conditions (i.e., medium and temperature). In addition, the use of laparoscopic procedures has introduced new variables, including the use of distension medium (e.g., carbon dioxide), causing a higher than normal intra-abdominal pressure that can potentially affect baroreceptors and modulate molecular pathways. Finally, the effects of prenephrectomy renal artery embolization in patients with large renal masses should be taken into consideration.

Drs. Marston Linehan and Gennady Bratslavsky at the Urologic Oncology Branch of the NCI are currently conducting an initiative to standardize RCC tissue procurement techniques. To this end, a form is used to systematically record the variables used in each surgery (Fig. 1). Importantly, the creation of a database of surgical variables will allow performing retrospective studies aimed at determining the effect of each variable on data accuracy.

In an additional effort to recognize and eliminate bias in the analysis of kidney tissue, Dr. Bratslavsky is leading a research project focused at defining the extent to which temperature and time of storage affect gene and protein expression of surgically removed renal tissue. In this prospective study, kidney tumors from patients with von Hippel Lindau (VHL) disease are used because of their relatively homogeneous morphologic features. The study design is outlined in Fig. 2. This project is critical to understanding the potential effect of storage and processing conditions on molecular analyses of renal masses and will likely facilitate the establishment of standard operating procedures.


Renal Tumor Procurement

Date_____ Lab Number_____ MR#_____

Last Name_____ First Name_____ MI_____

Clinical Dx_____ Specimen site_____

Induction Time/Anesthesia Start_____

Type of Anesthesia_____

Incision Time_____ Surgery type (lap, lap-assisted, open)

Clamped (Start_____ End_____ time_____)(Warm /Cold)/Unclamped

Tumor#_____ Label_____

Time resected_____ Time removed_____ Tumor Dimensions
____X____X____

Thin walled cyst <30% Solid 30-70% Solid >70% Solid

Path notes: Specimen Dimensions ____X____X____ ____% Procured

Piece#___ Time Frozen/Fixed_____ Bx? (yes / no) In vivo (yes / no)

Piece#___ Time Frozen/Fixed_____ Bx? (yes / no) In vivo (yes / no)

Fig. 1. Variables recorded in the NCI Tissue Procurement Form include clinical diagnosis, specimen site, type of anesthesia, induction/anesthesia start time, incision time, type of surgery (open versus laparoscopic), ischemia time, type of ischemia (warm versus cold), and resection time. Specific tumor characteristics such as size and macroscopic features are also included.

Image-guided techniques. A major challenge in the development of targeted therapies is the *in vivo* evaluation of target inhibition. The demonstration that the preclinical activity of a novel agent can be attributed to the modulation of the intended target in patients is a very valuable step in early phase clinical trials. Indeed, such a step can enable early determination of the optimal biochemical/biologically effective dose, which might be different from the maximum tolerated dose. In addition, such biopsies can also provide tissue to enable the determination of whether lack of efficacy of a specific agent is due to failure to modulate the intended target or, if the target is modulated as anticipated, a failure to identify a critical and/or clinically relevant target.

Computed tomography remains the gold standard procedure for guidance of tumor biopsies. Importantly, computed tomography with i.v. contrast medium seems critical in reducing vascular complications by visualizing vascular structures and, if possible, should be done in every biopsy procedure.

Unfortunately, the experience with needle biopsies of renal tumors is still very limited. Lechevallier et al. (3) studied the role of fine-needle biopsy with helical computed tomography guidance in the histopathologic evaluation of renal masses. Seventy-three renal biopsies were done and biopsy material was insufficient for histopathologic analysis in 15 (21%) procedures. No substantial morbidity occurred, but a small hematoma developed in 28 cases. No tumor seeding along the needle track was observed. The rate of failure was significantly higher in tumors <3.0 cm than in tumors >3.0 cm, (37% versus 9%; $P < 0.006$). Rybicki et al. (4), however, showed the decrease in diagnostic accuracy once tumors become >6 cm. Such decrease in diagnostic accuracy is most likely due to the presence of necrosis in larger masses.

Dr. Haaga recently conducted a retrospective study on 26 patients with renal tumors (tumor diameter, 0.9-20 cm) who underwent biopsy at Case Western Reserve University for diagnostic purposes. Twenty-three biopsies contained cancer including 21 renal primary tumors and 2 metastatic lesions. Of the three biopsies without cancer, one seemed to be a benign tumor and two were entirely necrotic. In line with the observation by Rybicki et al. (4), Dr. Haaga's data show that paradoxically larger tumors tend to have higher biopsy failure rates because of widespread necrosis. Interestingly, a recent literature review from the past 30 years published by Volpe et al. (5) concluded that the diagnostic accuracy of renal biopsy is close to 90%, is safe, and carries the minimal risk of the spread. Many of the studies, however, suffer from the lack of long-term follow-up information with regard to the oncologic safety of percutaneous renal biopsy. Therefore, a prospective study with a long-term follow-up would be welcomed and could address many outstanding issues.

Quality Control of Specimen Collection, Processing, Storage, and Review

Quality control of specimen collection. It is well-recognized that assessment of activation of key signaling molecules can provide important information on the activation status of molecular pathways in tissues. Active forms of kinases are usually detected with antibodies that selectively recognize

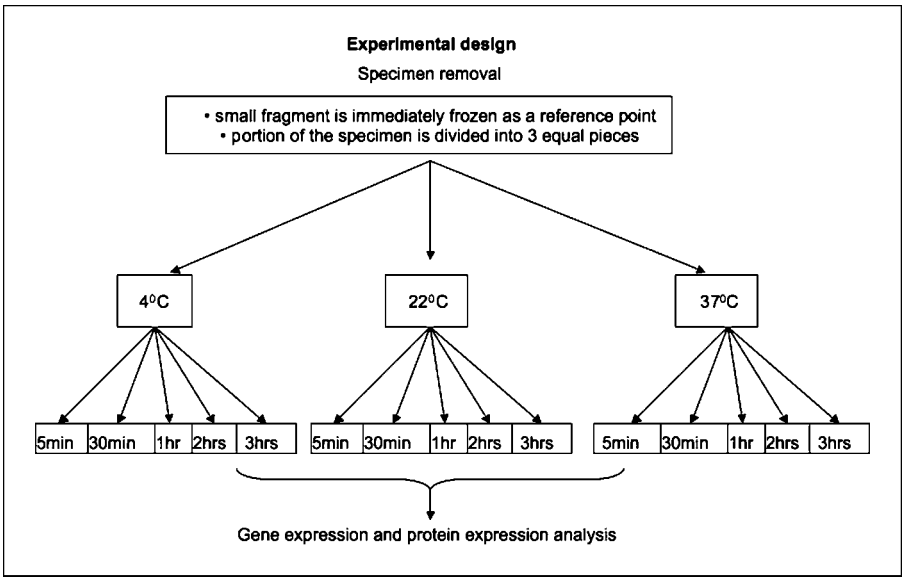


Fig. 2. Schematic of the experimental design used by Dr. Bratslavsky to assess the effect of storage temperature and time on mRNA and protein expression in renal cancer tissue.

phosphopeptides generated by autophosphorylation or by phosphorylation of their substrates. As phosphorylation and dephosphorylation events are known to occur very rapidly, there is the general concern that tissue acquisition procedures can bias the analyses by inducing significant changes in the phosphorylation status of potential biomarkers.

To address this concern, the Division of Cancer Treatment and Diagnosis Phase 0 Group at NCI used an animal model to compare the differential effects on protein expression and phosphorylation of the following: (*a*) "cryobiopsy" (a procedure in which tissue is frozen in liquid nitrogen before removal of the biopsy needle from the tumor mass); (*b*) surgical resection; (*c*) fine-needle aspirate; and (*d*) resection after sacrifice. To minimize variables in the different procedures, the same anesthesia method was used for the first three procedures. Findings showed considerable variability in phospho-AKT expression among the different tissue acquisition methods. Acquisition of tissue via cryobiopsy consistently allowed the detection of the highest levels of phospho-AKT. These results indicate that for some proteins in which the phosphorylation status is of interest, the acquisition method is crucial and comparison among tissue samples obtained by different methods should be done with caution.

Banking frozen tissue. Kidney cancer tissue banking requires collaborative efforts among pathologists, urologists, clinical research coordinators, and research scientists. Pathologic diagnosis remains first priority and, for this reason, tissue collection, processing, and distribution for research purposes should always be coordinated by pathologists. Indeed, the direct involvement of pathologists in all the phases of the banking process not only ensures that adequate tissue specimens are used for research studies but also guarantees that patient care is not adversely affected.

All tissue banking activities require approval from the Institutional Review Board. The Institutional Review Board should review the procedures for storing tissues into the bank as well as the procedures for release of stored tissues to investigators. We recommend that for each study, the protocol and copy of the Institutional Review Board approval are both submitted to the Pathology Department. Notification that an individual patient is on a given study should also be provided to pathologists before specimen collection.

As the biorepository of available tissues increases, and the need for retrospective specimen analysis in search of specific targets or biomarkers becomes more critical, multiple ethical considerations may come into play when analyzing specimens from patients with kidney cancer. For instance, testing for genes involved in hereditary RCC potentially affects patients' family members and raises concerns about privacy and genetic discrimination. Another issue relates to the accessibility of both tissue and clinical information after a given protocol is closed.

Further discussion on these kidney cancer–specific issues related to human subjects research is required to resolve situations raising ethical dilemmas.

Procesessing paraffin-embedded tissue. Variability in handling and processing of paraffin-embedded tissue specimens also represent important issues, especially in the analysis of clinical trials samples. The NCI is currently sponsoring various initiatives aimed at developing standard operating procedures for collection, handling, and storage of tissues from some types of cancers, including breast and prostate cancer. Various times of exposure to formalin, xylenes, as well as different temperatures during the tissue processing cycles have been shown to potentially affect the immunoreactivity of the tissue. Standardization of tissue processing steps would be extremely useful but it is very difficult to achieve, especially in nonacademic institutions where the majority of nephrectomies for primary renal cancers are done. Importantly, as an alternative and more practical approach, variability in processing protocols could be overcome by the development of new technologies.

In RCC, unique issues related to the high degree of tissue heterogeneity need to be addressed. Indeed, RCC is not a single disease but includes genetically and pathologically well-defined subtypes (6). Moreover, areas exhibiting different growth patterns (e.g., alveolar and sarcomatoid) and/or different cytologic features (e.g., clear cell and granular) are frequently observed within the same lesion. Molecular analyses of multiple areas from the same lesion will need to be done to assess the extent to which genetic and epigenetic heterogeneity within a given tumor contributes to phenotypic heterogeneity.

Novel high-throughput platforms (e.g., Affymetrix Exon Arrays, DASL) that will allow profiling of paraffin-embedded tissues will be invaluable for this purpose.

Gene expression profiling of archival tissue is also likely to facilitate identification of sets of genes that have prognostic and/or predictive value in RCC. Importantly, this effort might eventually lead to the development of a multigene assay that, similar to the Oncotype DX in breast cancer (7), will be able to provide a quantitative evaluation of the likelihood of cancer recurrence and also assesses the likely benefit from specific therapeutic approaches.

Pathology review issues. A clearer understanding of the genetic alterations associated with both hereditary and sporadic RCCs has led to a classification of renal neoplasms that is based on both pathologic and molecular tumor characteristics (6, 8). Importantly, such classification allows a better prediction of the clinical behavior of a tumor. Variants such as "sarcomatoid" and "granular" RCC are no longer recognized as separate entities as they simply describe cytologic characteristics of tumor cells that can be frequently observed in multiple RCC histotypes. Renal neoplasms that either do not fit in any of the recognized categories or have composite features are included in the "unclassified" group. Dr. Victor Reuter recently reevaluated of a series of 46 unclassified renal tumors using both morphologic and molecular variables. Interestingly, 26% were found to be clear cell and 13% chromophobe RCC. According to the latest classification, unclassified RCCs still represent 5% to 7% of all renal epithelial tumors.

The identification of genotypic and phenotypic characteristics of RCC subtypes has opened novel avenues for diagnosis and therapy. For instance, in clear cell carcinoma, VHL inactivation and subsequent hypoxia-inducible factor stabilization leads to the expression of downstream targets including vascular endothelial growth factor (VEGF), CAIX, and Glut-1, which can be used as tissue biomarkers for diagnostic and prognostic purposes, therapeutic targets, or predictors of therapeutic outcome. One of the major challenges, however, remains the variation of expression of such markers according to the tumor grade and their up-regulation in areas of tissue hypoxia, regardless of the VHL mutational status of the cancer. Prospective clinical trials will help clarify whether the expression levels of these markers can be reliably used to predict responses to therapies targeting the VHL–hypoxia-inducible factor pathway (i.e., sunitinib, sorafenib, and bevacizumab).

Improvement in early diagnosis has progressively led to identification of patients with small renal masses. As a consequence, observation of small solid renal masses is more frequently used (9). Although at this time, such nonoperative approach is more common in elderly and fragile patients, the ability to classify renal epithelial tumors *in vivo* is likely to play an important role in choosing therapeutic options in the near future for a wider population of patients. Development of novel imaging technologies as well as the use of needle biopsies or aspirates for pathologic diagnosis will be essential to move the field in this direction.

Issues Related to Analysis of Paraffin-Embedded Tissues

Preparation and use of tissue microarrays. Tissue microarrays (TMA) have been extensively used in biomarker development. As candidate biomarkers are increasingly identified by high-throughput genomic analyses, TMAs represent very powerful tools for validation studies (10).

The major concern regarding the use of TMAs is the representation of tissue heterogeneity. Indeed, because 0.6-mm tissue cores are usually used, there is the possibility that this small amount of tissue might not be representative of the whole tumor. Although whole tissue sections are considered the "gold standard," validation studies done in various types of cancers comparing immunohistochemical results in TMA and whole tissue sections (10) have shown that redundancy (2-10 cores per tumor) on the TMA decreases error introduced by limited sampling. However, there is general consensus that array construction protocols should be carefully developed for each tissue/tumor type.

In renal cancer, the construction of TMAs seems to be particularly challenging because of the relatively large size and the highly heterogeneous nature of the tumors. To address this issue, the Tissue Acquisition Pathology and Clinical Data Core of the Dana-Farber/Harvard Cancer Center Kidney Cancer Specialized Programs of Research Excellence has developed a RCC TMA construction protocol in which representative areas are selected on the basis of the Fuhrman Nuclear Grade (11). Specifically, for each tumor, two cores of the predominant and two cores of the highest Fuhrman Nuclear Grade are sampled. The inclusion of areas of both predominant and highest Fuhrman Nuclear Grade allows biomarker analysis in morphologically distinct areas of the tumor. Moreover, the use of a standardized protocol greatly facilitates meaningful comparisons between data obtained from different patient cohorts.

In collaboration with Dr. Meredith Regan, Dr. Signoretti's group recently did some validation studies comparing CAIX immunohistochemistry results obtained in whole tissue sections to those obtained in a TMA created according to the above-described protocol. The TMA contained cores from 60 RCCs obtained from patients treated with interleukin 2 in whom high CAIX tumor levels had been previously shown to be associated with response to therapy (12). By using the percentage of CAIX-positive cells (mean percentage of the four cores) as a continuous variable, a very good correlation was seen for tumors that were either very high or very low CAIX expressors. However, the mean percentage seemed to underestimate CAIX levels compared with the whole section in the tumors that showed intermediate expression (40-80% range). When the comparison was done using the previously established 85% cutoff, 10 of 60 (17%) cases were discordant, but the direction of the discordance seemed to be nonsystematic. Importantly, patients responding to interleukin-2 were found to have higher CAIX-expressing tumors compared with nonresponders both in the whole sections analysis [odds ratio, 2.99 (95% confidence interval, 0.86-11.2); $P = 0.065$] and the TMA study [odds ratio, 2.38 (95% confidence interval, 0.68-9.0); $P = 0.17$].

In summary, the analysis shows that in a relatively small cohort of tumors, a concordance of 83% is achieved when comparing CAIX expression levels in whole tissue sections and TMA slides. Although such results are encouraging, it must be noted that similar validation studies conducted in other tumor types showed higher concordance rates (13). Therefore, validation of specific immunohistochemical assays on RCC TMAs is recommended before such assays are extensively used in analyses of relatively small cohorts (<100 patients).

A general issue in the use of TMAs is the preservation of tissue antigenicity after sectioning. To minimize the waste of tissue, laboratories tend to prepare large batches of TMA sections. Unfortunately, sections stored for a long time experience antigenic loss, most likely due to tissue oxidation. Recoating the slides in paraffin before storage and storage in a nitrogen chamber might represent valid approaches to circumventing this problem (14).

Optimizing Biomarkers for Targeted Therapies in RCC

Major challenges facing targeted therapies for solid tumors include the selection of the patients who are most likely to benefit, documentation of target inhibition, and monitoring of response. As a consequence, clinically applicable biomarkers are needed to make significant advances in the field.

An improved understanding of the molecular basis of RCC has recently led to the identification of various molecular targets in the *VHL* pathway (e.g.,VEGF, VEGFR2, and PDGFR) and the development of several therapeutic agents that are both more active and better tolerated than previously used chemotherapy and immunotherapy approaches (15, 16). Indeed, tyrosine kinase inhibitors, including sorafenib, sunitinib, and axitinib, show clear activity in patients with advanced RCC. Both sorafenib and sunitinib have recently been approved by the U.S Food and Drug Administration for the treatment of patients with advanced kidney cancer. It is important to note that these drugs represent the first Food and Drug Administration–approved treatments for this type of cancer in more than a decade. Inhibitors of the mammalian target of rapamycin have also shown sufficient efficacy in clinical trials to prompt Food and Drug Administration approval of single agent temsirolimus in patients with advance kidney cancer (17–19).

Research by Thomas and colleagues (20) indicates that loss of *VHL* sensitizes both cells and xenografts to temsirolimus, and that the growth arrest caused by this agent correlates with a block in translation of mRNA encoding hypoxia-inducible factor (*HIF1A*). Importantly, VHL-deficient xenografts seem to display increased uptake of the positron emission tomography tracer fluorodeoxyglucose in an mammalian target of rapamycin–dependent manner. Such results strongly suggest that the *VHL* mutational status of renal tumors might predict for response to mammalian target of rapamycin inhibitors, and that fluorodeoxyglucose-positron emission tomography scans could be useful pharmacodynamic biomarkers in patients. More recently, Cho et al. (21) showed that both phospho-S6 ribosomal protein and phospho-Akt expression are promising predictive biomarkers for response to temsirolimus.

As the identification of patients who are likely to respond to a given treatment is a very important step toward the development of personalized medicine, mammalian target of rapamycin inhibitors should be now evaluated in biomarker-driven clinical trials. Specifically, putative markers of response should be assessed in biopsy specimens obtained before treatment and then monitored after therapy and potentially at the time of relapse. Thus, a critical first step in this process is the optimization of molecular assays to detect biallelic *VHL* inactivation as well as expression of activated phospho-AKT and p-S6 in clinical material. Molecular tests for *VHL* mutations, loss of heterozygosity, and promoter methylation in limited biopsy material are feasible but also impractical, time consuming, and expensive. Therefore, it would be extremely important to identify immunohistochemical markers that can be used as surrogates of VHL inactivation status in formalin-fixed, paraffin-embedded clinical samples.

Genomic Studies

Molecular studies in RCC. Large-scale molecular analyses in RCC have the potential of leading to the discovery of new markers for assessing risk, diagnosis, prognosis, response to therapy (tumor and host), and metastatic load.

Waldman and colleagues are working toward understanding and potentially overcoming challenges related to the analyses of DNA and RNA extracted from formalin-fixed paraffin-embedded tissues. Several genomic analyses, including direct sequencing of candidate cancer genes, need to be conducted on purified tumor cell populations isolated by microdissection. As a consequence, methods of amplifying nanogram quantities of DNA obtained through this procedure are often required. Pilot experiments showed that, unfortunately, DNA extracted form formalin-fixed paraffin-embedded tissue produced consistently low yields, by both isothermal Klenow polymerase-based and Phi29 DNA polymerase-based whole genome amplification techniques. In summary, optimal protocols for genomic studies of microdissected archival samples need to be developed.

RNA extraction from formalin-fixed paraffin-embedded tissues has also been evaluated by Waldman and colleagues using various commercially available technologies that were shown to produce different RNA yields. Importantly, a good correlation (Sperman Rank correlation coefficient, $r \geq 0.70$) between transcript levels in frozen and formalin-fixed paraffin-embedded samples was observed for six of the nine genes that were tested by quantitative PCR. These data suggest that mRNA quantification in archival tissue specimens is feasible and can be potentially applied to large series of kidney tumors.

Current efforts are devoted to predicting response to VEGF pathway–targeted therapy. A pilot study of 43 primary clear cell RCC from patients with metastatic disease treated with VEGF-targeted therapy (sunitinib, axitinib, or IFN/bevacizumab) was recently conducted by Dr. Waldman's group (22). Twenty-six patients (60%) had evidence of *VHL* mutation or promoter methylation. Patients with *VHL* mutation or methylation had an objective response rate of 48% compared with 35% in patients with no alterations. Interestingly, patients with severe *VHL* alterations (i.e., methylation or a mutation predicted to truncate or shift the reading frame) had a median time to progression of 13.3 months versus 7.4 months in patients with none of these features ($P = 0.06$). More recently, relative expression levels of candidate genes were measured in paraffin-embedded tissue from treated patients by quantitative reverse transcription-PCR and tested against clinical end points. Tumors with highest VEGF expression had median time to progression of 8.1 months versus 4.9 months for tumors with lowest expression ($P = 0.04$). Tumors with highest VEGFR1 expression had median time to progression of 13.3 months versus 6.4 months for tumors with lower expression ($P = 0.04$). Further studies are

needed to convincingly show that patients with alterations in *VHL* and the angiogenic pathways it regulates are more responsive to VEGF targeted treatments.

Transcriptional profiling. It is becoming increasingly evident that molecular classification of cancer is not only feasible but also opens new avenues for diagnostic evaluation and more rational therapeutic development. Several studies have convincingly shown that gene signatures can reliably differentiate malignant from morphologically normal kidney tissue and also separate different subtypes of RCC (23). In a recent analysis, Jones et al. (24) identified a 150-gene signature that is able to distinguish between the most common kidney tumor subtypes [clear cell RCC (cRCC), papillary RCC, chromophobe RCC, transitional cell carcinoma, and oncocytoma] with 100% accuracy. Further analyses will be critical to the identification of signaling pathway alterations that are either common or specific to RCC subtypes.

cRCC has an extremely variable and unpredictable natural history. Survival of patients with cRCC is limited by the development of metastases, which occur in 30% to 50% of cases (25, 26). Therefore, early identification of patients at risk for metastases would be an important prerequisite for selection of patients for aggressive adjuvant treatment and may eventually lead to new therapeutic approaches. Transcriptional profiling has been used to develop cRCC-specific outcome signatures. Recently, Jones et al. (24) derived a metastatic gene signature by comparing stage T_1 tumors and those with distant metastases. This signature has been successfully validated and seems to differentiate primary cRCCs associated with clinically detectable distant metastases at the time of nephrectomy from primary cRCCs without metastases. Overall, these data seem to indicate that patients with distant metastases at presentation might have a biologically distinct form of cRCC that can be identified by their gene expression pattern. How this pattern relates to that of renal cancer specimens in patients that only develop visible metastases after nephrectomy remains to be determined. Although much progress has been achieved to date, further studies on large multiinstitutional cohorts are absolutely necessary to address these issues and identify clinically relevant gene sets for RCC prognostication.

Assessment of Reproducibility of Assays Across Institutions

Interinstitutional reproducibility for molecular analysis of tissue is greatly influenced by many variables that can be categorized into preacquisition and postacquisition variables. Preacquisition variables include patient treatments (e.g., administration of drugs) before surgery as well variables related to surgical procedures such as vessel clamping time and anesthesia time. Postacquisition variables include tissue handling time and conditions from removal of tissue from patient to placement in fixative/freezing as well as freezing time and tissue processing conditions. Molecular characteristics are also influenced by short- and long-term storage conditions of both tissue specimens and purified biomolecules.

The effects of various storage conditions on mRNA stability were analyzed in a recent study from the Karolinska Institute (27). Fresh normal tonsil was cut into pieces and snap frozen either immediately or after being stored for 0.5, 1, 3, 6, or 16 hours at one of the following conditions: (*a*) room temperature, (*b*) on ice, (*c*) in normal saline, or (*d*) in a commercial RNA-stabilizing buffer (RNAlater). Surprisingly, when structural RNA integrity was analyzed by microchip electrophoresis, it was observed that the rRNA remained stable under all conditions for up to 16 hours. Transcript levels of specific genes (c-fos, hypoxia-inducible factor1α, Bcl2, proliferating cell nuclear antigen, transforming growth factor β1, and SMAD7) were also studied by real-time PCR for the different storage conditions and time points. mRNA levels were essentially stable when samples were kept on ice, whereas marked reduction of individual mRNAs was observed during storage at room temperature, in normal saline, and in RNAlater. It is through experiments such as this that it will be possible to generate data to develop evidence-driven standard operating procedures for handling tissue specimens to obtain accurate experimental data. As the effects of storage variables can be tissue specific, research projects such as the one that Dr. Bratslavsky is performing will be essential to clarifying the specific influence of temperature and time of storage on gene and protein expression in renal cancer specimens.

Conclusions

We have identified some major challenges in the use of tissue specimens for translational research in kidney cancer. The main topics meriting further discussion and/or investigation include the following: (*a*) establishment of standard operating procedures for kidney cancer tissue collection, processing, and storage; (*b*) development of standardized protocols for molecular assays widely used in kidney cancer research (e.g.,VHL genotyping and immunohistochemical staining for proteins in the VHL-hypoxia-inducible factor pathway); and (*c*) validation of current candidate biomarkers in a large multiinstitutional cohort.

Optimal procedures should be determined experimentally by comparing different protocols and/or methodologies. For instance, standard operating procedures should be developed using conditions shown to preserve the quality of the biomolecules of interest to the highest degree possible. To facilitate the accomplishment of this goal, we are planning to establish a Working Group that will be tasked to prioritize issues, design experiments, coordinate efforts, and identify new topics of discussion.

Efforts from the kidney cancer research community should be coordinated and integrated with existing NCI-supported initiatives devoted to defining guidelines for tissue-based research in other cancer types (e.g., the Inter–Specialized Programs of Research Excellence Prostate Biomarkers Study Group and the Breast International Group). Finally, as the NCI Best Practices for Biospecimen Resources report is now available; investigators in the kidney cancer research community are planning to work with member of the Office of Biorepositories and Biospecimen Research to ensure that protocols for kidney cancer tissue collection, processing, and analysis will be developed in compliance with NCI guidelines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Allan Pantuck, Christopher Wood, Brian Rini, and Ian Fore for actively participating in the Workshop.

References

1. Atkins MB, Ernstoff MS, Figlin RA, et al. Innovations and challenges in renal cell carcinoma: summary statement from the Second Cambridge Conference. Clin Cancer Res 2007;13:667–70s.

2. Brugarolas J. Renal-cell carcinoma-molecular pathways and therapies. N Engl J Med 2007;356:185–7.

3. Lechevallier E, Andre M, Barriol D, et al. Fine-needle percutaneous biopsy of renal masses with helical CT guidance. Radiology 2000;216:506–10.

4. Rybicki FJ, Shu KM, Cibas ES, Fielding JR, vanSonnenberg E, Silverman SG. Percutaneous biopsy of renal masses: sensitivity and negative predictive value stratified by clinical setting and size of masses. AJR Am J Roentgenol 2003;180:1281–7.

5. Volpe A, Kachura JR, Geddie WR, et al. Techniques, safety and accuracy of sampling of renal tumors by fine needle aspiration and core biopsy. J Urol 2007; 178:379–86.

6. Lopez-Beltran A, Scarpelli M, Montironi R, Kirkali Z. 2004 WHO classification of the renal tumors of the adults. Eur Urol 2006;49:798–805.

7. Kaklamani V. A genetic signature can predict prognosis and response to therapy in breast cancer: Oncotype DX. Expert Rev Mol Diagn 2006;6:803–9.

8. Reuter VE. The pathology of renal epithelial neoplasms. Semin Oncol 2006;33:534–43.

9. Chawla SN, Crispen PL, Hanlon AL, Greenberg RE, Chen DY, Uzzo RG. The natural history of observed enhancing renal masses: meta-analysis and review of the world literature. J Urol 2006;175:425–31.

10. Giltnane JM, Rimm DL. Technology insight: Identification of biomarkers with tissue microarray technology. Nat Clin Pract Oncol 2004;1:104–11.

11. Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. Am J Surg Pathol 1982;6:655–63.

12. Atkins M, Regan M, McDermott D, et al. Carbonic anhydrase IX expression predicts outcome of interleukin 2 therapy for renal cancer. Clin Cancer Res 2005; 11:3714–21.

13. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80:1943–9.

14. DiVito KA, Charette LA, Rimm DL, Camp RL. Long-term preservation of antigenicity on tissue microarrays. Lab Invest 2004;84:1071–8.

15. Feldman DR, Motzer RJ. Novel targets and therapies for metastatic renal cell carcinoma. Oncology (Huntingt) 2006;20:1745–53; discussion 1756.

16. Patel PH, Chadalavada RS, Chaganti RS, Motzer RJ. Targeting von Hippel-Lindau pathway in renal cell carcinoma. Clin Cancer Res 2006;12:7215–20.

17. Hudes G, Carducci M, Tomczak P, et al. Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med 2007;356:2271–81.

18. Hampton T. Trials probe new agents for kidney cancer. JAMA 2006;296:155–7.

19. Cho D, Signoretti S, Regan M, Mier JW, Atkins MB. The role of mammalian target of rapamycin inhibitors in the treatment of advanced renal cancer. Clin Cancer Res 2007;13:758–63s.

20. Thomas GV, Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med 2006;12:122–7.

21. Cho D, Signoretti S, Dabora S, et al. Potential histologic and molecular predictors of response to temsirolimus in patients with advanced renal cell carcinoma. Clin Genitourin Cancer 2007;5:379–85.

22. Rini BI, Jaeger E, Weinberg V, et al. Clinical response to therapy targeted at vascular endothelial growth factor in metastatic renal cell carcinoma: impact of patient characteristics and Von Hippel-Lindau gene status. BJU Int 2006;98:756–62.

23. Jones J, Libermann TA. Genomics of renal cell cancer: the biology behind and the therapy ahead. Clin Cancer Res 2007;13:685–92s.

24. Jones J, Otu H, Spentzos D, et al. Gene signatures of progression and metastasis in renal cell cancer. Clin Cancer Res 2005;11:5730–9.

25. Motzer RJ, Bacik J, Mazumdar M. Prognostic factors for survival of patients with stage IV renal cell carcinoma: memorial sloan-kettering cancer center experience. Clin Cancer Res 2004;10:6302–3S.

26. Cohen HT, McGovern FJ. Renal-cell carcinoma. N Engl J Med 2005;353:2477–90.

27. Micke P, Ohshima M, Tahmasebpoor S, et al. Biobanking of fresh frozen tissue: RNA is stable in nonfixed surgical specimens. Lab Invest 2006;86: 202–11.

Clinical Cancer Research

Tissue-Based Research in Kidney Cancer: Current Challenges and Future Directions

Sabina Signoretti, Gennady Bratslavsky, Frederick M. Waldman, et al.

Clin Cancer Res 2008;14:3699-3705.