RESEARCH ARTICLE

A Multidisciplinary Biospecimen Bank of Renal Cell Carcinomas Compatible with Discovery Platforms at Mayo Clinic, Scottsdale, Arizona

Thai H. Ho1 *, Rafael Nunez Nateras2, Huihuang Yan3, Jin G. Park4, Sally Jensen5, Chad Borges9, Jeong Heon Lee6, Mia D. Champion7, Raoul Tibes1, Alan H. Bryce1, Estrella M. Carballido1, Mark A. Todd8, Richard W. Joseph9, William W. Wong10, Alexander S. Parker11, Melissa L. Stanton12, Erik P. Castle2

1 Division of Hematology and Oncology, Mayo Clinic, Scottsdale, Arizona, United States of America, 2 Department of Urology, Mayo Clinic Hospital, Phoenix, Arizona, United States of America, 3 Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, Minnesota, United States of America, 4 Center for Personalized Diagnostics, Biodesign Institute, Arizona State University, Tempe, Arizona, United States of America, 5 Department of Chemistry & Biochemistry, The Biodesign Institute-Center for Personalized Diagnostics, Arizona State University, Tempe, Arizona, United States of America, 6 Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, United States of America, 7 Division of Biomedical Statistics and Informatics, Mayo Clinic, Scottsdale, Arizona, United States of America, 8 Division of Anatomic Pathology, Mayo Clinic, Scottsdale, Arizona, United States of America, 9 Division of Hematology and Oncology, Mayo Clinic, Jacksonville, Florida, United States of America, 10 Department of Radiation Oncology, Mayo Clinic, Scottsdale, Arizona, United States of America, 11 Departments of Health Sciences Research, Mayo Clinic, Jacksonville, Florida, United States of America, 12 Department of Laboratory Medicine/Pathology, Mayo Clinic, Scottsdale, Arizona, United States of America

* ho.thai@mayo.edu

Abstract

To address the need to study frozen clinical specimens using next-generation RNA, DNA, chromatin immunoprecipitation (ChIP) sequencing and protein analyses, we developed a biobank work flow to prospectively collect biospecimens from patients with renal cell carcinoma (RCC). We describe our standard operating procedures and work flow to annotate pathologic results and clinical outcomes. We report quality control outcomes and nucleic acid yields of our RCC submissions (N=16) to The Cancer Genome Atlas (TCGA) project, as well as newer discovery platforms, by describing mass spectrometry analysis of albumin oxidation in plasma and 6 ChIP sequencing libraries generated from nephrectomy specimens after histone H3 lysine 36 trimethylation (H3K36me3) immunoprecipitation. From June 1, 2010, through January 1, 2013, we enrolled 328 patients with RCC. Our mean (SD) TCGA RNA integrity numbers (RINs) were 8.1 (0.8) for papillary RCC, with a 12.5% overall rate of sample disqualification for RIN<7. Banked plasma had significantly less albumin oxidation (by mass spectrometry analysis) than plasma kept at 25°C (P<.001). For ChIP sequencing, the FastQC score for average read quality was at least 30 for 91% to 95% of paired-end reads. In parallel, we analyzed frozen tissue by RNA sequencing; after genome alignment, only 0.2% to 0.4% of total reads failed the default quality check steps of Bowtie2, which was comparable to the disqualification
ratio (0.1%) of the 786-O RCC cell line that was prepared under optimal RNA isolation conditions. The overall correlation coefficients for gene expression between Mayo Clinic vs TCGA tissues ranged from 0.75 to 0.82. These data support the generation of high-quality nucleic acids for genomic analyses from banked RCC. Importantly, the protocol does not interfere with routine clinical care. Collections over defined time points during disease treatment further enhance collaborative efforts to integrate genomic information with outcomes.

Introduction

To support medical research, institutional biobanking efforts have encompassed archived formalin-fixed, paraffin-embedded (FFPE) tissue blocks, frozen tissues, peripheral blood, questionnaires, and medical records [1–4]. Genitourinary diseases are heterogeneous and range from benign to malignant conditions [5,6]. The construction of a biobank encompassing both benign and malignant genitourinary diseases may yield clues regarding molecular progression of disease. In 2010, Mayo Clinic (Scottsdale, Arizona) initiated the Multidisciplinary Genitourinary Diseases Biospecimen Bank to prospectively collect biospecimens from patients with genitourinary diseases and to support health-related research. Herein, we report our experience with frozen banking protocols for renal cell carcinoma (RCC) biospecimens that are compatible with standard clinical practices.

The Cancer Genome Atlas (TCGA) Research Network has analyzed the DNA, RNA, and protein from various human tumors to generate molecular profiles, identify recurrent molecular aberrations, and create public data portals to support medical research [7–11]. Currently, TCGA integrates data from DNA methylation at CpG islands, microarray-based measurement of copy number, whole-exome sequencing, RNA sequencing, and reverse-phase protein arrays. The development of next-generation DNA, RNA, chromatin immunoprecipitation (ChIP) sequencing, and high-throughput protein analyses provides an opportunity for institutions to characterize genomic, transcriptomic, and proteomic alterations. However, the application of established and emerging platforms for molecular analysis depends on well-annotated and high-quality biospecimens. Furthermore, these platforms may necessitate large quantities of frozen biospecimens, requiring standard operating procedures to be in place at the time of tissue collection.

To address an unmet need for banked clinical specimens suitable for high-throughput genomic analysis, we developed a protocol to collect frozen tissue specimens, blood, and urine from patients affected by RCC through longitudinal biospecimen collection during follow-up appointments. We describe our standard operating procedures, workflow, and research coordinator efforts, as well as our quality assessment of biospecimens submitted to TCGA from a single institution. We also assessed the albumin oxidation in banked plasma, a surrogate of plasma quality. It is unknown whether banked specimens collected under routine clinical care are compatible with emerging discovery platforms such as ChIP sequencing. Herein, we demonstrate that our biobanking protocol is compatible with multiple platforms, including DNA, RNA, and ChIP sequencing, as well as mass spectrometry (MS) analysis.

Materials and Methods

Patient Eligibility and Recruitment

Patients eligible for enrollment were those seen at Mayo Clinic (Scottsdale, Arizona) who were ≥18 years old, able to provide informed consent, and undergoing evaluation for genitourinary...
diseases (kidney, urothelial, prostate, testicular, and penile malignancies or benign prostatic hyperplasia or nephrolithiasis). Patients were contacted during routine clinical visits or in preoperative settings within Mayo Clinic departments and divisions, including urology, radiation oncology, pathology, and medical oncology. Patients were excluded if they declined to participate or if the banking of their biospecimens would compromise the availability of tissue for diagnosis and standard clinical care. The protocol for collecting biospecimens was approved by the Mayo Clinic Institutional Review Board. The current informed consent form (S1 File), which was updated in 2012, was approved by the Mayo Clinic Institutional Review Board (protocol no. 08–000980). Patients were enrolled from June 1, 2010, through January 1, 2013. Patients provided written consent and the signed consent form was scanned into the electronic medical record. Patients also received a copy of the signed consent form. This consent procedure was approved by the Mayo Clinic Institutional Review Board.

**Blood and Urine Collection**

Blood and urine specimens are collected from patients preoperatively, postoperatively, and during systemic treatments (every 3 months) for routine clinical care. The blood and urine are transferred from the phlebotomist to the laboratory processing area within 30 minutes of collection. Blood is collected in BD Vacutainer tubes (red top, Fisher Scientific 02-685-112, no anticoagulants; lavender top, Fisher Scientific 02-657-32, K2 EDTA). Contents of red-top tubes are allowed to clot for 10 to 30 minutes, then centrifuged at 1,882 relative centrifugal force (RCF) for 10 minutes, and serum is transferred into 3×1.8-mL Nunc cryovials (Fisher Scientific 12565167N). Lavender-top tubes are centrifuged at 1,882 RCF in a refrigerated centrifuge; plasma is then divided and placed into 3×1.8-mL Nunc cryovials (Fisher Scientific 12565167N); the remaining buffy coat is separated into 3×1.8-mL Nunc cryovials (Fisher Scientific 12565167N). This protocol yields approximately 4.5 mL of plasma, serum, and buffy coat per collection visit. Samples are stored at −80°C.

For urine collections, 2 mL of urine is divided into 2 cryovials for storage; the remaining urine is centrifuged at 1,882 RCF for 5 minutes in a Hycor KOVA (Fisher Scientific VT87139) tube in a refrigerated centrifuge. The supernatant is removed and the pellet resuspended in 1 mL of urine and stored in a cryovial. Centrifuged samples are stored at −80°C.

**Tissue Collection**

Surgical specimens are handled according to standards of clinical care. The specimens are delivered directly from the operating room to the pathology department. After immediate macroscopic review, the pathologist decides whether adequate tissue is available for banking. The time from the start of warm ischemia to freezing can be as short as 10 minutes but as long as 110 minutes. The mean warm-ischemia time (ligation of blood flow) to freezing was 45.8 minutes. The warm-ischemia time is influenced by factors such as partial vs radical nephrectomy or an open vs minimally invasive approach. The tumor sample (and a sample of uninvolved tissue, if available) are macrodissected from the surgical specimen. Each sample is bisected, with 1 FFPE half as a mirror image representing the frozen biobank sample. The mirror-image FFPE sample is used as one of the clinical diagnostic slides. The biobank samples are immediately frozen in 7-mL polycarbonate tubes (Sarstedt 71.9923.610) in a −90°C bath of Novac engineered fluid (3M HFE-7000) cooled in a Histochill freezing bath (SP Scientific HC80A0). No embedding medium or preservative is added to frozen tissue. Tumor cellularity of the FFPE mirror image is assessed by a pathologist and reported as part of the standard-of-care pathology synoptic report.
Biospecimen Storage

Unique identification numbers are assigned to each individual container with bar code labels. Biospecimens are stored in a Brooks BioStore −80°C Automated Sample Storage System (Brooks Automation Inc). Samples can be retrieved manually or robotically.

Biobank Research Electronic Data Capture Database

A coded and secure Research Electronic Data Capture (REDCap) database was constructed with pertinent clinical information and database identifiers. Biospecimens are tracked by unique identification numbers assigned by the Research and Laboratory Information Management System (RLIMS). Pertinent pre-, peri-, and postoperative clinical variables (procedures, complications, and surgical outcomes) are annotated and linked to biospecimen data. In oncologic cases, we also annotate progression, survival, and treatment outcomes from the patient medical record. Clinical follow-up is maintained by data coordinators for matched blood, urine, and tissue (primary nephrectomies, biopsies, and metastectomies).

TCGA Papillary RCC Sample Submission

For papillary RCC solicited by TCGA (KIRP cases), a genitourinary pathologist (M.L.S.) reviewed the diagnostic slide representing the frozen tumor. Specimens were selected only if they met the standard TCGA biospecimen criteria with a corresponding buffy coat (for germline DNA analysis). The minimum DNA, RNA, and germline DNA yield required was 6.9 μg, 5.15 μg, and 4.9 μg, respectively. For reverse-phase protein array analysis, at least 10 mg of frozen tumor was required. Standard TCGA criteria included: 1) tumor sample composed predominantly of histologically viable-appearing tumor cells with ≥60% tumor nuclei; 2) ≤20% necrosis of sample volume; and 3) tumor weight ≥100 mg. The Mayo Clinic Institutional Review Board protocol was subsequently modified to reflect the shipment of samples to TCGA’s Biospecimen Core Resource (The Research Institute, Nationwide Children’s Hospital, Columbus, Ohio). Prior to shipping, protected health information was removed from all materials (specimen containers, diagnostic slides, pathology reports). The top slide submitted for review was the diagnostic slide cut from the bisected tumor, representing both the frozen tumor and the FFPE tumor. The initial enrollment, case quality control, other malignancy, and follow-up forms were completed by data coordinators using clinicopathologic variables entered from the REDCap database. Prism (v6.02; GraphPad Software, Inc [commercial software]) was used to generate scatter plots and statistics.

Analysis of Albumin and Apolipoprotein A-I Oxidation

Albumin S-cysteinylation and apolipoprotein A-I (apoA-I) methionine oxidation were analyzed as described in detail elsewhere [12]. Briefly, samples were thawed, mixed, and then centrifuged to sediment particulates. One-half microliter was removed and diluted 1,000-fold in 0.1% (v/v) trifluoroacetic acid. Five microliters of this solution were injected without delay onto a liquid chromatography—electrospray ionization—mass spectrometry instrument (Eksigent nanoLC-1D connected to a Bruker MicrOTOF-Q operating in time-of-flight only mode) equipped with a protein CapTrap (Optimize Technologies) rather than a conventional column. After flushing small molecules to waste for 3 minutes, albumin and apoA-I were eluted into the mass spectrometer by increasing the acetonitrile concentration over the CapTrap.

Mass spectra containing protein charge envelopes were averaged across approximately 1 minute and then charge-deconvoluted with DataAnalysis (v3.4; Bruker Corp [commercial software]) to a mass range of 1,000 Da on either side of any identified peak. Baseline values
were subtracted from deconvoluted spectra and all peak heights were calculated. Tabulated MS peak heights were exported to a spreadsheet for further calculation. The fractional abundance of S-cysteinylated (oxidized) albumin was determined by dividing the height of the MS peak representing S-cysteinylated albumin by the sum of the peak heights for native and S-cysteinylated albumin. ApoA-I methionine oxidation was calculated in an analogous fashion. No MS peaks representing apoA-I with 2 or 3 oxides were detected; however, if they had been, these peaks would have been weighted to calculate total weighted oxidation, as described elsewhere [12].

ChIP Sequencing

Nephrectomy samples underwent gross macrodissection to ensure that they contained >60% to 70% tumor, with verification by a mirror slide stained with hematoxylin-eosin. Frozen tissues were divided into 50-mg aliquots and stored in 1.5-mL centrifuge tubes at −70°C. Frozen tissues were homogenized on ice for 15–30 seconds in 500 μL 1× phosphate-buffered saline using a tissue grinder (AgileGrinder; ACTGene, Inc). Tissue homogenates were cross-linked using formaldehyde (final concentration, 1%) for 10 minutes; reactions were quenched with 125 mM glycine for 5 minutes at room temperature and washed once with Tris-buffered saline. Pellets were resuspended in cell lysis buffer (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 0.5% NP-40) and incubated on ice for 10 minutes. Lysates were divided into 2 aliquots and washed with micrococcal nuclease digestion buffer (20 mM Tris-HCl, pH 7.5; 15 mM NaCl; 60 mM KCl; 1 mM CaCl$_2$). After resuspending in 500 μL MNase digestion buffer containing a proteinase inhibitor cocktail (no. 37491; Active Motif Corp), lysates were incubated in the presence of 1,000 gel units of MNase (no. M0247S; New England Biolabs, Inc) at 37°C for 20 minutes with continuous mixing in a thermal mixer. After adding the same volume of sonication buffer (100 mM Tris-HCl, pH 8.1; 20 mM EDTA; 200 mM NaCl; 2% Triton X-100; 0.2% sodium deoxycholate), lysates were sonicated for 15 minutes (30 seconds on, 30 seconds off) using a Bioruptor Twin (model UCD-400; Diagenode Inc) and centrifuged at 21,130×g for 10 minutes. The cleared supernatant (equivalent to 10–20 mg of tissue) was incubated with 2 μg rabbit polyclonal anti—histone H3 lysine 36 trimethylation (H3K36me3) antibody (no. 61101, Active Motif Corp) on a rocker overnight. After adding 30 μL of protein G—agarose beads, reactions were further incubated for 3 hours. Beads were extensively washed with ChIP buffer (50 mM Tris-HCl, pH 8.1; 10 mM EDTA; 100 mM NaCl; 1% Triton X-100; 0.1% sodium deoxycholate), high-salt buffer (50 mM Tris-HCl, pH 8.1; 10 mM EDTA; 500 mM NaCl; 1% Triton X-100; 0.1% sodium deoxycholate), LiCl$_2$ buffer (10 mM Tris-HCl, pH 8.0; 0.25 M LiCl$_2$; 0.5% NP-40; 0.5% sodium deoxycholate; 1 mM EDTA), and Tris-EDTA buffer. Bound chromatin was eluted and reverse—cross-linked at 65°C overnight. DNA was purified using a MinElute PCR purification kit (no. 28004; Qiagen Inc) after RNase A and proteinase K treatment. H3K36me3 chromatin immunoprecipitation was validated by performing quantitative PCR in the genomic loci targeting the ACTB gene body (positive control) and the neighboring intergenic region (negative control). ChIP quantitative PCR was carried out in triplicate on indicated genomic regions using SYBR Green Supermix (Bio-Rad Laboratories, Inc). The following primer sequences were used: hActin: F 5’-CCTCATGCGCTTGGCACA; hActin: R 5’-GCCCTTCTGACTGTCTTCCT; hCh19-intergenic: F 5’-AGCTTTGCTTTTCTCCAAAGTTACTC; hCh19-intergenic: R 5’-TAGCTTGTCGACACCTCAGAGA. The comparative ΔCt method was used to determine relative enrichment compared with input.

ChIP-seq libraries were then prepared from 10 ng ChIP and input DNA using the Ovation Ultralow DR Multiplex kit (NuGEN Technologies Inc). ChIP-seq libraries were sequenced to 51 base pairs (bp) from both ends on an Illumina HiSeq 2000 instrument.
Sequence data were analyzed by the Mayo Clinic Center for Individualized Medicine Bioinformatics Program. The ChIP-Seq pipeline version 2 integrates open-source software packages to analyze ChIP sequencing data and identify profiles from chromatin regulators, posttranslational histone modifications, and transcription factor binding [13]. The source code is publicly available at http://bioinformaticstools.mayo.edu/research/hichipseq-pipeline/, with hyperlinks corresponding to individual software packages. The main features include 1) read-quality checking; 2) read mapping and filtering; 3) library quality assessment; 4) peak calling analysis; and 5) data visualization (S1 Fig). FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (publicly available software) was used to assess the 51-bp paired-end reads for the average quality score per base position. The Burrows-Wheeler Alignment tool (http://bio-bwa.sourceforge.net/) (publicly available software) was used to map the paired-end reads to the reference genome (hg19) [14]. SICER, a package developed for scoring broad binding events (http://home.gwu.edu/~wpeng/Software.htm) (publicly available software), was used to identify H3K36me3 peaks [15]. To plot the genome-wide enrichment, we split the human genome into nonoverlapping 500-bp windows, estimated the number of mapped fragments overlapping each window, and normalized to the number of fragments per 10 million mapped pairs.

To profile the level of H3K36me3 occupancy within the gene-body, we downloaded the RefGene annotation from the UCSC Genome Browser table browser (http://genome.ucsc.edu/cgi-bin/hgTables) and intersected that with peak coordinates using BEDTools (http://bedtools.readthedocs.org/en/latest/) (publicly available) [16].

RNA Sequencing of Frozen Nephrectomy Specimens

Frozen nephrectomy tissue samples (25–30 mg) were pulverized using a hammer and placed in lysis reagent (Qiazol, no. 79306; Qiagen Inc). RNA from the pulverized tissue was isolated using the miRNeasy Mini Kit (no. 217004; Qiagen Inc) on the QIAcube instrument (no. 9001292; Qiagen Inc). RNA libraries were prepared according to the manufacturer’s instructions for the Encore NGS Library Systems (NuGEN Technologies Inc). Briefly, first-strand cDNA was generated from total RNA using DNA/RNA chimeric primers and reverse transcriptase, creating a cDNA/RNA hybrid. The second-strand cDNA was then synthesized from the DNA/RNA duplex. The resulting double-stranded cDNA was amplified by Single Primer Isothermal Amplification (NuGEN Technologies Inc) using a chimeric primer, DNA polymerase, and RNase H. After amplification, products were modified by random priming and extension to create double-stranded products that were suitable for generating libraries for sequencing. The double-stranded products (200 ng) were fragmented in an ultrasonicator (E210; Covaris Inc) for 5 minutes to generate ~150-bp fragments, which then underwent blunt-end repair. Illumina-compatible paired-end index adapters were ligated to the 5’ and 3’ ends of each fragment. The adapter-modified cDNA fragments were enriched with 5 cycles of PCR amplification. The concentration and size distribution of the resulting libraries were determined by using a Bioanalyzer DNA 1000 chip (Agilent Technologies, Inc). The concentration values were confirmed by Qubit fluorometry (Life Technologies Corp). Libraries were loaded onto Illumina TruSeq v3 paired-end flow cells at concentrations of 8–10 pM to generate cluster densities of 600,000–800,000/mm² following Illumina’s standard protocol and using the Illumina cBot and paired-end cluster kit version 3. Flow cells were sequenced as 100×2 paired-end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and HiSeq Control (version 2.0.12.0; Illumina, Inc [commercial software]). Base-calling was performed using Real-Time (version 1.17.21.3; Illumina, Inc [commercial analysis software]).

After proper permissions were obtained, RNA sequencing data on normal and tumor kidney samples were downloaded from TCGA kidney cancer (https://tcga-data.nci.nih.gov/tcga/);
Kidney Renal Cell Carcinoma [KIRC] dataset. Sequencing data have been submitted to Gene Expression Omnibus, accession number GSE69198. For meta-analyses, we first normalized reads per kilobase of transcript per million reads mapped (RPKM) values from our frozen-tissue RNA sequencing data, with the standard normalization method of TCGA, ie, \( \text{RPKM} \div \text{75th percentile RPKM value} \times 1,000 \).

**Results**

**Biobanking Work Flow and Pathologic Characteristics of the RCC Biobank Specimens**

Patients undergoing evaluation for genitourinary diseases were recruited and provided consent during urology, radiation oncology, pathology, or medical oncology appointments. Pathologic characteristics of the cohort enrolled from June 1, 2010, to January 1, 2013, are summarized in Table 1. The biobank represents earlier T stages of RCC, consistent with surgical treatments for localized disease. Fig 1 illustrates the work flow for consent of patients, collection and processing of biospecimens, and data input into the centralized REDCap database. To accommodate tissues from diverse anatomical sites, we developed a generalized work flow for collection of frozen tissue, blood, and urine. For tissue, the priority is for diagnostic testing for purposes of routine clinical care, with the rest of the tissue frozen at \(-80^\circ\text{C}\) in a manner compatible with TCGA tissue collection requirements [17,18]. The protocol is compatible with collection of tissue at the time of surgical resection or biopsy at the primary site (nephrectomy) and at distant metastatic sites.

| Characteristic                  | Value          |
|--------------------------------|----------------|
| Age, median (range), y         | 66 (32–92)     |
| Sex, No. (%)                   |                |
| Male                           | 115 (35)       |
| Female                         | 213 (65)       |
| Cancer T stage, No. (%)        |                |
| T1a                            | 176 (54)       |
| T1b                            | 67 (20)        |
| T2a                            | 15 (5)         |
| T2b                            | 7 (2)          |
| T3a                            | 40 (12)        |
| T3b                            | 13 (4)         |
| T3c                            | 3 (1)          |
| T4                             | 7 (2)          |
| Pathology, No. (%)             |                |
| Clear cell                     | 216 (66)       |
| Papillary                      | 36 (11)        |
| Oncocytoma                     | 36 (11)        |
| Chromophobe                    | 20 (6)         |
| Angiomyolipoma                 | 13 (4)         |
| Othera                         | 7 (2)          |

*Includes unclassified, mucinous, and tubular spindle cell carcinoma.

doi:10.1371/journal.pone.0132831.t001
TCGA Specimen Submission and Quality Control

Frozen papillary RCC samples were submitted for processing to TCGA. As a control, buffy coat collected concomitant with tissue was submitted as a germline control. The genitourinary pathologist reviewed the diagnostic slide, which is a mirror image of the frozen tissue and FFPE, to ensure that samples met tumor-specific pathology requirements. The mean (SD) tumor weight was 376 (130) mg. DNA and RNA yields from tumor and matched buffy coat are listed in Table 2. Representative diagnostic slides were sent to TCGA (Fig 2). All extracted DNA samples passed the TCGA requirements. With our work flow, 2 of 16 submitted tumors (12.5%) did not meet RNA integrity number (RIN) requirements (RIN < 7). Our mean (SD) TCGA RINs were 8.1 (0.8) for papillary RCC. Table 3 lists the genomic platforms used to analyze TCGA samples with publicly accessible data.

Analysis of Albumin and apoA-I Oxidation as a Metric of Plasma Specimen Integrity

As previously reported [12], albumin and apoA-I in human blood plasma are susceptible to oxidative S-cysteinylolation and methionine sulfoxidation, respectively, when plasma is exposed

Table 2. DNA and RNA Yields From Samples Submitted to The Cancer Genome Atlas.

| Sample          | Yield, mean (SD), μg |
|-----------------|----------------------|
| Tumor DNA       | 28.6 (14.9)          |
| Buffy coat DNA  | 48.0 (22.4)          |
| Tumor RNA       | 46.9 (10.3)          |

* The mean (SD) tumor weight was 376 (130) mg.
to temperatures above its freezing point of −30°C. At both room temperature and −20°C, albumin oxidation (S-cysteinylation) increases as per the complement of an exponential decay function before reaching a plateau (for most samples) at a relative fractional abundance of about 0.4. ApoA-I methionine oxidation proceeds at a slower pace, requiring days of exposure to room temperature or months of storage at −20°C [12]. On average, the banked samples from patients with RCC described in this study had albumin oxidation (S-cysteinylation) levels consistent with several hours’ exposure to room temperature and no long-term storage above

Fig 2. Papillary Renal Cell Carcinoma (RCC) Samples Submitted to The Cancer Genome Atlas (TCGA). A, Representative mirror images of top slides sent for independent pathology review to the Biospecimen Core Resource of TCGA. H&E indicates hematoxylin-eosin stain; original magnification x10 (left) and x40 (right). B, Scatterplot of TCGA quality assessment of RNA processed from papillary RCC specimens. The black horizontal dotted line represents the mean, with the vertical error bars representing the SD. RNA integrity numbers (RINs) were assessed by TCGA RNA bioanalyzer. Two samples with RIN <7 were disqualified.

doi:10.1371/journal.pone.0132831.g002
−30°C (Fig 3). Consistent with this observation, apoA-I oxidation remained below quantifiable levels for tested samples.

**H3K36me3 Profiling by ChIP Sequencing**

We tested the utility of our banked specimens for emerging technologies such as ChIP sequencing that require larger amounts of frozen tissue (>100 mg). Altered chromatin accessibility is observed in clear cell RCC and is associated with alterations of H3K36me3 [19–22]. Compared to other genome-wide discovery platforms, ChIP sequencing often requires larger amounts of tissue (>100 mg). To determine whether our biobanking work flow for specimens was compatible with H3K36me3 ChIP sequencing, we performed ChIP quantitative polymerase chain reaction to ensure quality and yield of immunoprecipitated chromatin from 6 samples. An H3K36me3 antibody was used for immunoprecipitation of endogenous H3K36me3-bound DNA. Since H3K36me3 is enriched at exons and active genes, polymerase chain reaction primers were optimized for active genes, such as actin, and for an intergenic region (negative control) [23,24]. At a minimum, 4-fold enrichment is required before proceeding to sequencing [25,26]. We found that actin was enriched 8- to 32-fold over the intergenic controls (Fig 4), which indicated that the quality and yield of immunoprecipitated DNA/chromatin complexes were sufficient for generating ChIP-sequencing libraries. S2 Fig shows the optimal 100–400 bp DNA fragments that were compatible with ChIP sequencing.

To evaluate the properties of ChIP-sequencing libraries derived from banked frozen nephrectomy samples, we checked paired-end read quality, genome-wide enrichment level, and distribution of H3K36me3 occupancy. Both reads had good quality, with FastQC scores of 32 to 39 across all 51 bp (Fig 5A and 5B). The first 3 bp had the lowest quality score for both reads, with the first read having slightly better sequencing quality, as is typically seen for paired-end sequencing. In addition, over 95% of the first reads had an average quality score of ≥30 compared to 91% to 92% of the second reads. Only a small proportion of the reads had poor quality scores (≤20, on average): <0.5% for the first read and <3% for the second,
respectively (data not shown). Each library had 27.0 to 30.6 million pairs of raw reads, and 91.7% to 92.8% of the pairs mapped to the reference genome (hg19) by BWA. Of these, 85.4% to 86.7% represented uniquely mapped pairs.

For ChIP-sequencing experiments, another quality measurement is the level of enrichment between immunoprecipitated DNA and the input DNA control. The box plots represent the top 5% of the 500-bp windows with the highest number of fragments (Fig 5C). The peak regions from each H3K36me3 immunoprecipitated library showed an average of about 4-fold enrichment over the corresponding regions in the input library. In published ChIP-sequencing studies in cell lines, H3K36me3 is predominantly associated with gene bodies [23,27]. To map H3K36me3 in gene bodies, we examined the distribution of H3K36me3 across each chromosome. The 3 RCC libraries showed a 2% to 5% reduction of H3K36me3 occupancy over the gene body, compared to matched normal samples (Fig 5D). H3K36me3 is enriched at exons, and the difference between different chromosomes in H3K36me3 occupancy over the gene body largely reflected the ratio of exon sizes over gene sizes. For example, chromosome 19 had the highest size ratio of exon/gene (13.6%) and also the highest H3K36me3 coverage of gene body (47.4%-53.5%). Similar patterns were also found for chromosomes 16 and 22.

RNA Sequencing Quality of Frozen Nephrectomy Samples

To assess the quality of RNA sequencing data from frozen nephrectomy samples, we analyzed RNA from frozen tissue profiled in parallel by ChIP sequencing (N = 6). A representative bioanalyzer profile and RIN of isolated RNA from paired samples (tumor and normal) is depicted in S3 Fig. We first examined the sequencing quality per base for forward and reverse reads. As shown in S4 Fig, the average Phred score of each base in all reads was above 30, indicating a high-quality sequencing run. Next, we compared the quality of RNA sequencing from our frozen tissues with that of an RCC cell line (786-O), which was prepared under optimal RNA

Fig 3. Albumin Oxidation (S-Cysteinylation) Observed in the Banked Renal Cell Carcinoma (RCC) Plasma Samples. Control plasma was freshly collected from a nominally healthy donor, and albumin oxidation levels were observed at 25°C. Significantly less albumin oxidation was observed in samples from patients with RCC compared with albumin in plasma kept at 25°C for ≥17.5 hours (P<.001; Mann-Whitney U test). (Data from Borges CR et al [12].)
isolation conditions. After genome alignments, only 0.2%-0.4% of total reads from frozen tissues failed the default quality check steps of Bowtie2 (disqualified reads are shown in Table 4), which was comparable to the disqualification ratio (0.1%) of the 786-O cell line. The mapping ratios for our frozen tissues were slightly lower (76.4%-83.8%) than those from the cell line (84.5%).

One of the key metrics of RNA sequencing quality and coverage is the number of identified genes. To identify genes expressed in both the Mayo Clinic and TCGA cohorts with high confidence, we counted genes with normalized expression values \( \frac{C_2}{C_1} \approx 1.0 \) RPKM across all samples. As shown in Table 5, among ~20,500 protein-coding genes, we observed a similar number of expressed genes in the Mayo Clinic and TCGA tissues. Poor-quality RNA sequencing often results in biased amplification and consequent overrepresentation of a subset of genes. To assess the RNA sequencing quality, expression levels of the most abundant genes across all samples were examined. Fig 6A shows that TPT1, EEF1A1, B2M, and GPX3 were most abundant in both the Mayo Clinic and TCGA samples. With the exception of TPT1 (highly abundant in the Mayo Clinic samples and accounted for up to 9.5% of expression values of total normalized counts), we did not identify any other
overrepresented genes. Despite fundamental differences in sample source, sample processing, and sequencing library generation, the overall correlation coefficients between the Mayo Clinic vs TCGA tissues ranged from 0.75 to 0.82 (Fig 6B). Although expression levels of low-abundance genes were slightly overestimated in the Mayo Clinic samples, genes with mid to high
abundance were more linearly aligned. These data support the compatibility of our biobanking protocol with RNA and ChIP-sequencing platforms for frozen RCC.

Discussion
The Multidisciplinary Genitourinary Diseases Biospecimen Bank in Arizona is a collaboration among investigators in urology, radiation oncology, pathology, and medical oncology to study the spectrum of benign and malignant genitourinary disease. Our work flow suggests that our biobanking protocol for RCC does not interfere with routine clinical care and is compatible with systematic cancer genomics projects such as TCGA and MS analysis of plasma, as well as with newer technologies (eg, ChIP sequencing) requiring larger quantities of frozen tissue. Herein, we reported the quality control of our TCGA submissions for papillary RCC (paired frozen tissue, mirror slide, and buffy coat), MS analysis of albumin S-cysteinylation as a surrogate of plasma integrity, and ChIP sequencing analysis of frozen tissues.

With matched temporal collections of frozen tissue and buffy coat, we can link molecular characterizations of DNA, RNA, and protein to treatment outcomes. The annotation of tumor cellularity of matched FFPE specimens in the pathology report expedites the identification of tumor blocks suitable for targeted exome sequencing, and immunohistochemistry [28–30]. Integration of the clinical information into a REDCap database facilitates collaborations to query specific disease cohorts linked to outcomes data.

Table 4. Comparison of Mapped Reads Between Frozen Nephrectomy Samples and a Renal Cell Carcinoma Cell Line (786-O).  

| Source and Sample ID | Total Reads | Disqualified Reads | Unmapped Reads | Mapped Reads |
|----------------------|-------------|--------------------|----------------|--------------|
|                      | Count       | % Total            | Count          | % Total      | Count          | % Total      |
| Kidney tumor         |             |                    |                |              |                |              |
| ccRCC1               | 414,293,024 | 1,801,533 0.4      | 79,723,359 19.2| 332,768,132 80.3|
| Uninv kidney 1       | 421,101,208 | 1,018,419 0.2      | 68,354,681 16.2| 351,728,108 83.5|
| ccRCC2               | 427,575,250 | 1,535,886 0.4      | 99,270,525 23.2| 326,768,839 76.4|
| Uninv kidney 2       | 426,317,444 | 1,205,535 0.3      | 67,952,300 15.9| 357,159,609 83.8|
| ccRCC3               | 409,282,398 | 1,565,821 0.4      | 92,300,799 22.6| 315,415,778 77.1|
| Uninv kidney 3       | 424,633,572 | 878,592 0.2        | 75,578,922 17.8| 348,176,058 82.0|
| Cell line            | 164,760,980 | 149,964 0.1        | 25,449,107 15.4| 139,161,909 84.5|

Abbreviations: ccRCC1, clear cell renal cell carcinoma 1; ccRCC2, clear cell renal cell carcinoma 2; ccRCC3, clear cell renal cell carcinoma 3; Uninv, uninvolved.

doi:10.1371/journal.pone.0132831.t004

Table 5. Comparison of Mapped Reads Between the Mayo Clinic and TCGA Nephrectomy Samples.

| Sample Group          | No. of Samples | No. of Genes With Normalized Score ≥ 128, Mean (SD) |
|-----------------------|----------------|---------------------------------------------------|
| Mayo kidney tumor     | 6              | 12,177.0 (317.2)                                  |
| TCGA kidney tumor     | 533             | 11,339.8 (324.8)                                  |
| TCGA normal kidney    | 72              | 11,444.5 (172.9)                                  |

Abbreviation: TCGA, The Cancer Genome Atlas.

doi:10.1371/journal.pone.0132831.t005
Fig 6. Expression Profiles and Correlation of Genes in the Mayo Clinic and TCGA Cohorts. A, Expression of the most abundant genes in Mayo Clinic tissues. Expression levels of the 30 most-abundant genes in frozen tumors (upper 6 bars) and TCGA kidney tumors (averaged from 553 samples) are shown in stacked bars, with percentages of total normalized counts. Reads for TPT1 occupied up to 9.5% of total reads. B, Correlation of gene expression between the Mayo Clinic and TCGA kidney tumors. Normalized expression values of a representative Mayo Clinic tumor and TCGA kidney tumor samples (averaged from 553 samples) were compared. Genes in the boxed area have normalized expression values >128. ccRCC1 indicates clear cell renal cell carcinoma 1; ccRCC2, clear cell renal cell carcinoma 2; ccRCC3, clear cell renal cell carcinoma 3; TCGA, The Cancer Genome Atlas; Uninv, uninvolved.

doi:10.1371/journal.pone.0132831.g006
Limitations

Our study has several limitations. First, our study represents biospecimens processed within 4 years of collection. The biobank started prospective collections in 2010, and our DNA and RNA yields, MS analysis, and ChIP sequencing may not be representative of samples stored longer than 4 years or of other genitourinary tumors (eg, urothelial, testicular, penile, or prostate cancers). For ChIP sequencing, we chose a covalent histone modification, H3K36me3, for enrichment of DNA; our H3K36me3 ChIP enrichment may not be comparable to ChIP enrichment using antibodies directed to transcription factors, which may not be as stable in banked frozen tissue. Our RNA yields reflect an overall mean warm-ischemia time of 45.8 minutes (range, 10–110 minutes) for the RCC specimens (N = 328), which was influenced by factors such as partial vs radical nephrectomy or an open vs a minimally invasive approach. Despite the warm-ischemia time, the overall rate of sample disqualification for RIN <7 for TCGA submissions was 12.5%. In a prior study, RNA degradation in nephrectomy specimens was observed primarily after 4 hours at 37°C, and prolonged warm ischemia was associated with changes in gene expression profiles [31]. Furthermore, we studied only nephrectomy specimens; other tissues may have different sensitivities to warm-ischemia times.

Second, we examined the compatibility only of frozen nephrectomy specimens, rather than archival FFPE nephrectomy specimens, with various discovery platforms. The use of FFPE has additional challenges that include fragmentation and degradation of nucleic acids, and alterations of epitopes during the fixation and deparaffinization process. FFPE-compatible DNA, RNA, and ChIP sequencing protocols for various tissues have been published, but further study is required to determine the compatibility of these protocols with FFPE nephrectomy tissue [32–34].

Third, elevated concentrations of circulating free cysteine and cystine (the disulfide dimer of free cysteine) have been reported in patients with severely compromised renal function [35]. Their effects on albumin S-cysteinylation in vivo are not entirely clear because the ratio of cysteine to cystine in blood does not vary much [36], presumably because the redox potential of blood plasma is rather tightly regulated. Ex vivo, however, elevated concentrations of cysteine and cystine in the presence of oxygen would almost certainly lead to higher-than-normal maximum levels of S-cysteinylated albumin through disulfide exchange with cystine and through sulfenic acid—mediated disulfide bond formation with cysteine [37–40]. In some patients with renal failure, the fractional abundance of S-cysteinylated albumin has been reported at over 50%, although these studies lacked detailed documentation of sample-handling conditions [41–44]. Essentially, this means that elevated ex vivo levels of albumin S-cysteinylation are likely to occur faster in patients with renal failure than in patients without elevated plasma cysteine or cystine.

Conclusion

Genitourinary diseases represent a spectrum of disease with varying phenotypes and genotypes. The increase in tumor molecular profiling has led to a need for integration of genotype-phenotype relationships with contemporary treatments (definitive surgical management, medical therapy, and/or radiation). The construction of a biobank that encompasses both benign and malignant genitourinary diseases may also elucidate the mechanism of the molecular progression of disease prior to overt clinical symptoms. Our multidisciplinary collaboration ensures longitudinal collection of matched tissue, blood, and urine during the course of standard care as well as during experimental treatments that will be available for projects that require access to pre- and/or postintervention time points. The prospective collection of frozen biospecimens allows future next-generation platforms to interrogate specimens linked to
outcomes. A comprehensive prospective collection also facilitates banking of frozen tumors with rare subtypes and histologies, which may not be identified until completion of dedicated immunohistochemical staining (ie, not intraoperatively). Furthermore, the comparison of benign and malignant tissue may yield clues regarding risk-based algorithms for personalized treatment. The elucidation of distinct molecular phenotypes of RCC will further improve upon the prognostic and therapeutic stratification of patients.

Supporting Information

S1 Fig. Flowchart of Mayo Clinic ChIP-Seq pipeline. The integrative analysis uses datasets from ChIP libraries and a reference genome. The main features include 1) read-quality checking; 2) read mapping and filtering; 3) library quality assessment; 4) peak calling analysis; and 5) data visualization. The source code is available at http://bioinformaticstools.mayo.edu/research/hichipseq-pipeline/. ChIP indicates chromatin immunoprecipitation; H3K36me3, histone H3 lysine 36 trimethylation.

S2 Fig. DNA Fragment Analysis of Nephrectomy ChIP Libraries and Input Chromatin. Before library preparation, the majority of input chromatin is from mononucleosomes with a DNA fragment size of 100–200 bp. After library preparation for ChIP sequencing, the target fragment size is 100–400 bp. bp indicates base pair; ccRCC1, clear cell renal cell carcinoma 1; ccRCC2, clear cell renal cell carcinoma 2; ccRCC3, clear cell renal cell carcinoma 3; ChIP, chromatin immunoprecipitation; H3K36me3, histone H3 lysine 36 trimethylation; Uninv, uninvolved.

S3 Fig. Bioanalyzer Data of RNA Isolated From Nephrectomy Tissue. A, ccRCC1 with RIN = 8.6. B, Uninvolved kidney with RIN = 8.6. The 28s/18s rRNA ratios are given, with gel images to the right. ccRCC1 indicates clear cell renal cell carcinoma 1; FU, fluorescence unit; RIN, RNA integrity number.

S4 Fig. Sequencing Quality Scores per Base. Phred scores per base for forward (A) and reverse (B) reads of a representative sample are shown. Red dotted lines represent the threshold (score = 30) for good-quality sequencing. Plots were generated by FASTQC software. Solid red lines indicate median values; solid blue lines, mean values; boxes, 25th to 75th percentiles.

S1 File. Consent Form for the Research Study.
Program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The Multidisciplinary Genitourinary Diseases Biospecimen Bank is supported financially in part by the Gloria A. and Thomas J. Dutson Jr. Kidney Research Endowment; and by the Center for Individualized Medicine, Mayo Clinic (Rochester, Minnesota).

Author Contributions
Conceived and designed the experiments: THH RNN JGP MLS WWW ASP EPC. Performed the experiments: THH AHB EMC MAT RWJ MLS. Analyzed the data: THH HY MDC JHL RT SJ CB JGP. Wrote the paper: THH RNN HY JGP SJ CB JHL MDC RT AHB EMC MAT RWJ MLS WWW ASP EPC.

References
1. Esgueva R, Park K, Kim R, Kitabayashi N, Barbieri CE, Dorsey PJ Jr, et al; Weill Cornell Medical College. Next-generation prostate cancer biobanking: toward a processing protocol amenable for the International Cancer Genome Consortium. Diagn Mol Pathol. 2012 Jun; 21(2):61–8. doi: 10.1097/PDM.0b013e31823b6da6. PMID: 22555088
2. Carmignani L, Picozzi S, Casellato S, Bozzini G, Marenghi C, Macchi A, et al. A proposed new technique in prostate cancer tissue bio-banking: our experience with a new protocol. Pathol Oncol Res. 2012 Jul; 18(3):663–8. Epub 2012 Jan 4. doi: 10.1007/s12253-011-9492-6 PMID: 22215310
3. Olson JE, Ryu E, Johnson KJ, Koenig BA, Maschke KJ, Morrisette JA, et al. The Mayo Clinic Biobank: a building block for individualized medicine. Mayo Clin Proc. 2013 Sep; 88(9):952–62. Erratum in: Mayo Clin Proc. 2014 Feb;89(2):276. doi: 10.1016/j.mayocp.2013.06.006 PMID: 24001487
4. Kang B, Park J, Cho S, Lee M, Kim N, Min H, et al. Current status, challenges, policies, and bioethics of biobanks. Genomics Inform. 2013 Dec; 11(4):211–7. Epub 2013 Dec 31. doi: 10.5808/GI.2013.11.4.211 PMID: 24465232
5. Wu W, Yang D, Tiselius HG, Ou L, Mai Z, Chen K, et al. Collection and storage of urine specimens for measurement of urolithiasis risk factors. Urology. 2015 Feb; 85(2):299–303. Epub 2014 Nov 1. doi: 10.1016/j.urology.2014.10.030 PMID: 25623670
6. Sun H, Sun R, Hao M, Wang Y, Zhang X, Liu Y, et al. Effect of duration of ex vivo ischemia time and storage period on RNA quality in biobanked human renal cell carcinoma tissue. Ann Surg Oncol. 2015 Jan 8. [Epub ahead of print].
7. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 2008 Oct 23; 455(7216):1061–8. Epub 2008 Sep 4. Erratum in: Nature. 2013 Feb 28;494(7438):506. doi: 10.1038/nature11252 PMID: 18772890
8. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. Nature. 2011 Jun 29; 474(7353):609–15. Erratum in: Nature. 2012 Oct 11;490(7419):288. doi: 10.1038/ nature10166 PMID: 21720365
9. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012 Jul 18; 487(7407):330–7. doi: 10.1038/nature11252 PMID: 22810696
10. Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers. Nature. 2012 Sep 27; 489(7417):519–25. Epub 2012 Sep 9. Erratum in: Nature. 2012 Nov 8;491(7423):288. doi: 10.1038/nature11404 PMID: 22960745
11. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013 Jul 4; 499(7456):43–9. Epub 2013 Jun 23. doi: 10.1038/ nature12222 PMID: 23792563
12. Borges CR, Rehder DS, Jensen S, Schaab MR, Sherba ND, Yassine H, et al. Elevated plasma albumin and apolipoprotein A-I oxidation under suboptimal specimen storage conditions. Mol Cell Proteomics. 2014 Jul; 13(7):1890–9. Epub 2014 Apr 15. doi: 10.1074/mcp.M114.038455 PMID: 24736286
13. Yan H, Evans J, Kalmbach M, Moore R, Middha S, Luban S, et al. HiChiP: a high-throughput pipeline for integrative analysis of ChiP-Seq data. BMC Bioinformatics. 2014 Aug 15; 15:280. doi: 10.1186/1471-2105-15-280 PMID: 25128017
14. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009 Jul 15; 25(14):1754–60. Epub 2009 May 18. doi: 10.1093/bioinformatics/btp324 PMID: 19451168
15. Zang C, Schones DE, Zeng C, Cui K, Zhao K, Peng W. A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. Bioinformatics. 2009 Aug 1; 25(15):1952–8. Epub 2009 Jun 8. doi: 10.1093/bioinformatics/btp340 PMID: 19505939

16. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics. 2010 Mar 15; 26(6):841–2. Epub 2010 Jan 28. doi: 10.1093/bioinformatics/btq033 PMID: 20110278

17. Andreasson A, Kiss NB, Christofer Juhlin C, Hoog A. Long-term storage of endocrine tissues at −80°C does not adversely affect RNA quality or overall histomorphology. Biopreserv Biobank. 2013 Dec; 11 (6):366–70. PMID: 24475321

18. Shabihkhani M, Lacey GM, Wei B, Mareninov S, Lou JJ, Vinters HV, et al. The procurement, storage, and quality assurance of frozen blood and tissue biospecimens in pathology, biopsypository, and biobank settings. Clin Biochem. 2014 Mar; 47(4–5):258–66. Epub 2014 Jan 12. doi: 10.1016/j.clinbiochem.2014.01.002 PMID: 24424103

19. Simon JM, Hacker KE, Singh D, Brannon AR, Parker JS, Weiser M, et al. Variation in chromatin accessibility in human kidney cancer links H3K36 methyltransferase loss with widespread RNA processing defects. Genome Res. 2014 Feb; 24(2):241–50. Epub 2013 Oct 24. doi: 10.1101/gr.158253.113 PMID: 24158655

20. Edmunds JW, Mahadevan LC, Clayton AL. Dynamic histone H3 methylation during gene induction: HYPB/Setd2 mediates all H3K36 trimethylation. EMBO J. 2008 Jan 23; 27(2):406–20. Epub 2007 Dec 20. PMID: 18157086

21. Dillon SC, Zhang X, Trievel RC, Cheng X. The SET-domain protein superfamily: protein lysine methyltransferases. Genome Biol. 2005; 6(8):227. Epub 2005 Aug 2. PMID: 16086857

22. Dalglish GL, Furge K, Greenman C, Chen L, Bignell G, Butler A, et al. Systematic sequencing of renal cell carcinoma reveals inactivation of histone modifying genes. Nature. 2010 Jan 21; 463(7279):360–3. Epub 2010 Jan 6. doi: 10.1038/nature08672 PMID: 20054297

23. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, et al. High-resolution profiling of histone methylations in the human genome. Cell. 2007 May 18; 129(4):823–37. PMID: 17512414

24. Kolacinska-Zwierz P, Down T, Latorre I, Liu T, Liu XS, Ahringer J. Differential chromatin marking of introns and expressed exons by H3K36me3. Nat Genet. 2009 Mar; 41(3):376–81. Epub 2009 Feb 1. doi: 10.1038/ng.322 PMID: 19182803

25. Haring M, Offermann S, Danker T, Horst I, Peterhansel C, Stam M. Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization. Plant Methods. 2007 Sep 24; 3:11. PMID: 17982552

26. Blecher-Gonen R, Barnett-Itzhaki Z, Jaitin D, Amann-Zalcenstein D, Lara-Astiaso D, Amit I. High-throughput chromatin immunoprecipitation for genome-wide mapping of in vivo protein-DNA interactions and epigenomic states. Nat Protoc. 2013 Mar; 8(3):539–54. Epub 2013 Feb 21. doi: 10.1038/nprot.2013.023 PMID: 23429716

27. Chantatit S, Depaux A, Hery P, Barral S, Thuret JY, Dimitrov S, et al. Histone H3 trimethylation at lysine 36 is associated with constitutive and facultative heterochromatin. Genome Res. 2011 Sep; 21 (9):1426–37. Epub 2011 Jul 29. doi: 10.1101/gr.118091.110 PMID: 21803857

28. Nunez-Nateras R, Castle EP, Protheroe CA, Stanton ML, Ocal TI, Ferrigni EN, et al. Predicting response to bacillus Calmette-Guérin (BCG) in patients with carcinoma in situ of the bladder. Urol Oncol. 2014 Jan; 32(1):45.e23–30. Epub 2013 Sep 18.

29. Choueiri TK, Fay AP, Gray KP, Callea M, Ho TH, Albignes L, et al. PD-L1 expression in nonclear-cell renal cell carcinoma. Ann Oncol. 2014 Nov; 25(11):2178–84. Epub 2014 Sep 5. doi: 10.1093/annonc/mdu445 PMID: 25193987

30. Ho TH, Park IY, Zhao H, Tong P, Champion MD, Yan H, et al. High-resolution profiling of histone H3 lysine 36 trimethylation in metastatic renal cell carcinoma. Oncogene. [HHS Public Access]. In press. p. 1–24.

31. Liu NW, Sanford T, Srinivasan R, Liu JL, Khurana K, Aprelikova O, et al. Impact of ischemia and procurement conditions on expression in renal cell carcinoma. Clin Cancer Res. 2013 Jan 1; 19 (1):42–9. Epub 2012 Nov 7. doi: 10.1158/1078-0432.CCR-12-2606 PMID: 2316194

32. Fanelli M, Amatori S, Barozzi I, Minucci S. Chromatin immunoprecipitation and high-throughput sequencing from paraffin-embedded pathology tissue. Nat Protoc. 2011 Nov 10; 6(12):1905–19. doi: 10.1038/nprot.2011.406 PMID: 22082985

33. Fanelli M, Amatori S, Barozzi I, Soncini M, Dal Zotto R, Bucci G, et al. Pathology tissue-chromatin immunoprecipitation, coupled with high-throughput sequencing, allows the epigenetic profiling of patient samples. Proc Natl Acad Sci U S A. 2010 Dec 14; 107(50):21535–40. Epub 2010 Nov 24. doi: 10.1073/pnas.1007647107 PMID: 21106758
34. Hedegaard J, Thorsen K, Lund MK, Hein AM, Hamilton-Dutoit SJ, Vang S, et al. Next-generation sequencing of RNA and DNA isolated from paired fresh-frozen and formalin-fixed paraffin-embedded samples of human cancer and normal tissue. PLoS One. 2014 May 30; 9(5):e98187. doi:10.1371/journal.pone.0098187 PMID: 24878701

35. Nakanishi T, Hasuike Y, Otaki Y, Hama Y, Nanami M, Miyagawa K, et al. Free cysteine is increased in plasma from hemodialysis patients. Kidney Int. 2003 Mar; 63(3):1137–40. PMID: 12631098

36. Jones DP, Carlson JL, Mody VC, Cai J, Lynn MJ, Sterberg P. Redox state of glutathione in human plasma. Free Radic Biol Med. 2000 Feb 15; 28(4):625–35. PMID: 10719244

37. Rehder DS, Borges CR. Cysteine sulfenic acid as an intermediate in disulfide bond formation and non-enzymatic protein folding. Biochemistry. 2010 Sep 7; 49(35):7748–55. doi:10.1021/bi1008694 PMID: 20712299

38. Turell L, Botti H, Carballal S, Ferrer-Sueta G, Souza JM, Duran R, et al. Reactivity of sulfenic acid in human serum albumin. Biochemistry. 2008 Jan 8; 47(1):358–67. Epub 2007 Dec 14. PMID: 18078330

39. Turell L, Botti H, Carballal S, Rada R, Alvarez B. Sulfenic acid: a key intermediate in albumin thiol oxidation. J Chromatogr B Analyt Technol Biomed Life Sci. 2009 Oct 15; 877(28):3384–92. Epub 2009 Mar 28. doi:10.1016/j.jchromb.2009.03.035 PMID: 19386559

40. Turell L, Carballal S, Botti H, Rada R, Alvarez B. Oxidation of the albumin thiol to sulfenic acid and its implications in the intravascular compartment. Braz J Med Biol Res. 2009 Apr; 42(4):305–11. PMID: 19330257

41. Soejima A, Matsuzawa N, Hayashi T, Kimura R, Ootsuka T, Fukuoka K, et al. Alteration of redox state of human serum albumin before and after hemodialysis. Blood Purif. 2004; 22(6):525–9. Epub 2004 Dec 3. PMID: 15583477

42. Bar-Or D, Heyborne KD, Bar-Or R, Rael LT, Winkler JV, Navot D. Cysteinylation of maternal plasma albumin and its association with intrauterine growth restriction. Prenat Diagn. 2005 Mar; 25(3):245–9. PMID: 15791656

43. Regazzoni L, Del Vecchio L, Altermare A, Yeum KJ, Cusi D, Locatelli F, et al. Human serum albumin cysteinylation is increased in end stage renal disease patients and reduced by hemodialysis: mass spectrometry studies. Free Radic Res. 2013 Mar; 47(3):172–80. Epub 2013 Jan 9. doi:10.3109/10715762.2012.756139 PMID: 23215783

44. Nagumo K, Tanaka M, Chuang VT, Setoyama H, Watanabe H, Yamada N, et al. Cys34-cysteinylated human serum albumin is a sensitive plasma marker in oxidative stress-related chronic diseases. PLoS One. 2014 Jan 8; 9(1):e85216. doi:10.1371/journal.pone.0085216 PMID: 24416365