The Somatic Genomic Landscape of Chromophobe Renal Cell Carcinoma


1Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX 77030, USA
2Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, CRC Room 1W-5940, Bethesda, MD 20892, USA
3Center for Biomedical Informatics, Harvard Medical School, Boston, MA 02115, USA
4The Eli and Edythe L. Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA 2142, USA
5USC Epigenome Center, University of Southern California, Los Angeles, CA 90033, USA
6Samsung Genome Institute, Samsung Medical Center, Seoul, Korea
7Curriculum in Genetics and Molecular Biology, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA
8Department of Molecular Biology and Biochemistry, Rutgers University, Busch Campus, Piscataway, NJ 08854, USA
9Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08903, USA
10National Institute of Environmental Health Sciences, 111 T.W. Alexander Drive, Research Triangle Park, NC 27709, USA
11Canada’s Michael Smith Genome Sciences Centre, BC Cancer Agency, Vancouver BC V5Z 4S6, Canada
12Department of Biology & Biochemistry, University of Houston, 4800 Calhoun Road, Houston, TX 77204, USA
13Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA
14Department of Pathology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
15Leidos Biomedical Research, Basic Science Program, Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA
16Department of Medicine, Weill-Cornell Medical College, New York, NY 10021, USA
17Department of Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
18Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, USA
19Department of Medicine, Harvard Medical School, Boston, MA 02215, USA
20Department of Surgery, Urology Service, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA
21Division of Genetics, Harvard Medical School, Boston, MA 02215, USA
22Division of Genetics, Brigham and Women’s Hospital, Boston, MA 02115, USA
23Department of Genetics, Yale School of Medicine, New Haven, CT 06520, USA
24Co-first author
25Co-senior author
*Correspondence: rathmell@med.unc.edu (W.K.R.), creighto@bcm.edu (C.J.C.)
http://dx.doi.org/10.1016/j.ccr.2014.07.014

SUMMARY

We describe the landscape of somatic genomic alterations of 66 chromophobe renal cell carcinomas (ChRCCs) on the basis of multidimensional and comprehensive characterization, including mtDNA and whole-genome sequencing. The result is consistent that ChRCC originates from the distal nephron compared with other kidney cancers with more proximal origins. Combined mtDNA and gene expression analysis implicates changes in mitochondrial function as a component of the disease biology, while suggesting...
alternative roles for mtDNA mutations in cancers relying on oxidative phosphorylation. Genomic rearrangements lead to recurrent structural breakpoints within TERT promoter region, which correlates with highly elevated TERT expression and manifestation of katanias, representing a mechanism of TERT upregulation in cancer distinct from previously observed amplifications and point mutations.

INTRODUCTION

Rare tumor types offer a unique opportunity to investigate and discover mechanisms of tumorigenesis. Chromophobe renal cell carcinoma (ChRCC) is a subtype of renal cell carcinoma (RCC), representing ~5% of this heterogeneous group of cancers arising from the nephron (Störkel et al., 1997), with 3,000 new cases annually in the United States (Jemal et al., 2013). Although ChRCC typically exhibits an indolent pattern of local growth, with greater than 90% 10-year cancer-specific survival (Amin et al., 2002; Przybycin et al., 2011), aggressive features and metastasis can occur. ChRCC is associated with a distinct aneuploidy pattern (Speicher et al., 1994); however, genome-wide evaluation of its somatic mutation spectrum has not been reported. ChRCC is associated with germline mutation of FLCN in the autosomal-dominant cancer predisposition Birt-Hogg-Dubé (BHD) syndrome, in which 34% of BHD-associated kidney tumors are ChRCC (Nickerson et al., 2002; Pavlovich et al., 2002; Schmidt et al., 2001), and with germline mutation of PTEN in Cowden syndrome (Shuch et al., 2013). Previous studies have suggested a nonglycolytic metabolic profile for ChRCC, using 18F-fluorodeoxyglucose positron emission tomography/computed tomography (Ho et al., 2012), and have shown that the genomic profile comprises unique whole-chromosome losses rather than focal events (Speicher et al., 1994).

Genomic profiling of rare cancers, such as ChRCC, can provide a more complete picture of the disease. Although very large sample numbers (>5,000) may be needed for some disease types in order to detect rare mutational events (Lawrence et al., 2014), in many cases, there remain undiscovered frequent mutations that drive disease. When data integration across multiple platforms is applied, patterns observed in one data type may be reflected in the other data types, building a more conclusive set of findings with regard to revealing driver events. For example, early DNA microarray studies of breast cancer, for example, globally assaying a single data type for 65 tumors (Pero et al., 2000) and incorporating clinical data, have had an enduring impact on our understanding of breast and other cancers, while PBRM1 mutations were discovered in clear cell kidney cancers from an initial analysis of just 25 tumors (Varela et al., 2011). Understudied cancers, such as ChRCC, may hold this potential for discovery as well.

RESULTS

Copy-Number and Whole-Exome Analysis

The Cancer Genome Atlas (TCGA) collected a total of 66 primary ChRCC specimens (Table S1 available online) with matching normal tissue/blood, in order to better characterize the molecular basis of this cancer using multiple data platforms (Table 1; Table S1). Our comprehensive analysis of ChRCC involved a systematic examination by data type, including copy number and whole-exome sequencing (WES). By SNP array analysis, loss of one copy of the entire chromosome, for most or all of chromosomes 1, 2, 6, 10, 13, and 17, was seen in the majority of cases (86%; Figure 1A). Losses of chromosomes 3, 5, 8, 9, 11, 18, and 21 were also noted at significant frequencies (12%–58%). There were no focal copy-number events by GISTIC analysis (Mermel et al., 2011), suggestive of a simpler chromosomal landscape for ChRCC in comparison with that of other cancers, including the more common clear cell type RCC (ccRCC). We subdivided our ChRCC cases according to previously defined histologic categories of “classic” (n = 47), which demonstrate the classical pale cytoplasmic features for which the disease was named, and “eosinophilic” (n = 19), based on abundant, eosinophilic cytoplasm and densely packed mitochondria, by expert consensus pathology review (Brunelli et al., 2005). Although all classic cases showed the characteristic ChRCC copy-number pattern, only about half of the eosinophilic cases (10 of 19) showed the same, with four eosinophilic cases showing no copy-number alterations. This suggests a degree of genomic heterogeneity that distinguishes the histopathology-based classifications.

WES of 66 ChRCC cases targeted ~186,260 exons in ~18,091 genes, achieving 90% target coverage at a minimum of 20x for both tumor and matched normal samples. Overall, ChRCC displayed a low median rate of exonic somatic mutations (~0.4 per Mb) compared with most tumors (Alexandrov et al., 2013), approximately 3-fold less than the median number seen in ccRCC (which differences were also observable within

Significance

Rare diseases can provide insights into the biology of more common pathologies. Using diverse molecular platforms, we deconstructed ChRCC, a tumor characterized by slow but persistent growth and high resistance to conventional cancer therapies. Global molecular patterns provide clues as to this cancer’s cell of origin. mtDNA alterations represent an integral component of the molecular portrait of ChRCC. The observed TERT promoter rearrangements may result from genomic instability in precancerous cells undergoing the crisis stage of immortalization, leading to activated telomerase. These data will facilitate further discovery of driver alterations extending beyond the exome as well as the generation of hypotheses that can advance our molecular understanding of this and other cancers.
strata defined by age or stage), with the one exception showing elevated somatic mutation rate (>10/Mb by WES) and mutation signature of DNA mismatch repair deficiency (Alexandrov et al., 2013). Using alternative sequencing instrumentation, we validated 60 somatic mutation events for a set of 30 genes both arising from WES and having inferred biologically relevance (Table S2). Although our lower case numbers limited purely data-driven approaches to assigning statistical significance to infrequently mutated genes, we did have sufficient power to identify significant genes with a frequency of \(\frac{C}{24}\) 10% (Lawrence et al., 2014). Only two significant genes were thus identified (MutSig q < 0.1): TP53 and PTEN.

TP53 was frequently mutated in 32% of cases (21 of the 66 profiled), with mutations correlating with decreased expression of p53 transcriptional targets (Figures S1A–S1C). PTEN was the next most frequently mutated, with 9% (6 of 66) nonsilent mutations detected. No other genes were found to be mutated at a frequency higher than 5%, though mutations involving cancer-relevant genes were found at lower frequencies (Figure 1B). Biological significance could be ascribed to infrequently mutated genes, in terms of associated pathways, including the p53 and PTEN pathways (Table S2).

DNA Methylation and RNA Analysis

TCGA data platforms allow for comparisons between tumor types (Cancer Genome Atlas Research Network et al., 2013). For example, we observed widespread differences in DNA methylation between ChRCC and ccRCC (Figure 2A), involving over 64,000 loci out of \(~450,000\) profiled (\(p < 0.001\), t test using logit-transformed data, beta value difference > 0.1). ChRCC displayed more hypomethylation and fewer hypermethylation events compared with ccRCC. We also observed epigenetic silencing of CDKN2A/p16 in four ChRCC cases (Figure 2B). In principle, differential DNA methylation patterns could involve

<table>
<thead>
<tr>
<th>Table 1. Summary of Data Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data Type</td>
</tr>
<tr>
<td>TCGA core sample set (n = 66 total cases)</td>
</tr>
<tr>
<td>Whole-genome DNA sequence</td>
</tr>
<tr>
<td>mtDNA sequence</td>
</tr>
<tr>
<td>DNA copy number/genotype</td>
</tr>
<tr>
<td>mRNA expression</td>
</tr>
<tr>
<td>Cpg DNA methylation</td>
</tr>
</tbody>
</table>

See also Table S1.

*To amplify mitochondrial DNA.

Figure 1. Gene Mutations and Copy Alterations in ChRCC

(A) Copy-number alterations (red, gain; blue, loss of one copy) by cytoband region (marker: darker color, p arm; lighter color, q arm) in ChRCC and ccRCC. Pathway-altered cases: p53, PTEN, Complex I.

(B) Somatic mutation: nuclear DNA; mtDNA (20-60% het); structural variant: eosin.

See also Figure S1 and Table S2.

DNA Methylation and RNA Analysis

TCGA data platforms allow for comparisons between tumor types (Cancer Genome Atlas Research Network et al., 2013). For example, we observed widespread differences in DNA methylation between ChRCC and ccRCC (Figure 2A), involving over 64,000 loci out of \(~450,000\) profiled (\(p < 0.001\), t test using logit-transformed data, beta value difference > 0.1). ChRCC displayed more hypomethylation and fewer hypermethylation events compared with ccRCC. We also observed epigenetic silencing of CDKN2A/p16 in four ChRCC cases (Figure 2B). In principle, differential DNA methylation patterns could involve
cancer-relevant pathways but may also reflect the cell of origin of the cancer (Shen and Laird, 2013). On the basis of immunohistochemical analyses (Prasad et al., 2007), ChRCC has been postulated to arise from intercalated cells in the distal convoluted tubule of the nephron, while ccRCC is thought to arise from cells in the proximal convoluted tubule; however, this issue has remained unresolved. The above DNA methylation patterns were consistent with distinct origins, leading us to further explore these origins using gene expression data.

We examined our gene expression data in the context of an external gene expression data set of normal tissue microdissected from various regions of the nephron (Cheval et al., 2012). On the basis of immunohistochemical analyses (Prasad et al., 2007), ChRCC has been postulated to arise from intercalated cells in the distal convoluted tubule of the nephron, while ccRCC is thought to arise from cells in the proximal convoluted tubule; however, this issue has remained unresolved. The above DNA methylation patterns were consistent with distinct origins, leading us to further explore these origins using gene expression data.

We examined our gene expression data in the context of an external gene expression data set of normal tissue microdissected from various regions of the nephron (Cheval et al., 2012). Supervised analysis, globally comparing each TCGA ChRCC or ccRCC tumor expression profile (n = 66 and n = 417, respectively) with that of each sample in the nephron atlas, showed high mRNA expression correlations for ChRCC with distal regions of the nephron. ccRCC gene expression, however, was correlated with patterns associated with the proximal nephron (Figure 2C). These associations were also evident when focusing on the subset of differential genes in ChRCC versus ccRCC associated with inverse DNA methylation changes (Figure 2D). These results put into context many of the widespread molecular differences between these two kidney cancer types, as well as suggesting that cancers may be defined in part by cell of origin in addition to genetic aberrations.

In addition to widespread differences in gene expression between ChRCC and ccRCC, and differences from normal kidney (Figure S2A and Table S3), unsupervised clustering of mRNA profiles indicated further molecular heterogeneity within ChRCC, with at least two subsets identified (Figure S2B) as defined by differential gene expression patterns. Cluster analysis of micro-RNA (miRNA) profiles also indicated heterogeneity (Figure S2C), and we could identify anticorrelations between miRNAs and their predicted mRNA targets (Table S4), including an anti-correlation (false discovery rate [FDR] < 0.01) involving miR-145 (low in ChRCC versus normal) and the complex I-associated NDUFA4 gene (Figure S2D) (Kano et al., 2010). Molecular correlates of patient survival in ChRCC were identifiable at levels of...
mRNA, miRNA, and DNA methylation (Table S5); many of these correlates were shared with those previously observed for ccRCC (Cancer Genome Atlas Research Network, 2013) and included cell cycle genes, but not the “Warburg effect”-like patterns of aggressive ccRCC (Cancer Genome Atlas Research Network, 2013).

Pathway and mtDNA Analysis

When viewed in the context of mitochondrial function, expression of nuclear-encoded genes in ChRCC, with compared to normal kidney, suggested increased utilization of the Krebs cycle and electron transport chain (ETC) for ATP generation (Figure 3A; Figures S3A and S3B). In ChRCC, nearly all genes encoding enzymes in the Krebs cycle showed increased expression over normal, with the entry of pyruvate into the Krebs cycle via acetyl coenzyme A likely through the pyruvate dehydrogenase complex. Concordantly, all complexes of the ETC demonstrated mRNA increases in at least one gene. These patterns could reflect an increased level of mitochondrial biosynthesis, resulting in greater numbers of mitochondria within each tumor cell; this possibility is supported by both the increased expression of mitochondrial biogenesis regulator PPARGC1A (possibility is supported by both the increased expression of mitochondrial biogenesis regulator PPARGC1A, and increased mitochondrial genome copy numbers (four times more on average in ChRCC versus normal kidney; Figure 3B; Figure S3C). These findings interestingly parallel the eosiophilic histology observed in some ChRCC, corresponding to the high uptake of eosin by mitochondria. Eosinophilic ChRCC tumors share many features with the benign variant oncocytoma, which is also characterized by dense accumulations of mitochondria (Amin et al., 2008; Tickoo et al., 2000). Furthermore, the gene expression landscape appeared very different from that of ccRCC, in which expression of genes involved in mitochondrial functions is strongly suppressed (Figure S3D) (Cancer Genome Atlas Research Network, 2013). These findings suggest that various bioenergetics strategies may support tumor growth and that not all cancers necessarily seek to minimize their reliance upon oxidative phosphorylation (Cancer Genome Atlas Research Network, 2013).

Given the indicated prevalent role of mitochondria in ChRCC and the likelihood of rapid mitochondrial genome replication (Figure 3B), we sequenced mtDNA from 61 of our 66 ChRCC cases, using a PCR-based amplification approach (Table S6). In all, we identified 142 somatic mutation events (i.e., not present in the normal) at various levels of heteroplasmy (i.e., mixture with other variants), 75 of these residing within the commonly altered D-loop noncoding region (Chatterjee et al., 2006). Thirty-five mutation events (involving 27 cases) were present in over 50% of mtDNA copies in the tumor (>50% heteroplasy) (Figure 4A). Human mtDNA encodes 13 proteins involved in respiration and oxidative phosphorylation (Figure 3A), and we found 15 nonsilent mutations in 12 ChRCC cases involving these genes (>50% heteroplasy), all of which validated using alternative strategies, including whole-genome sequencing (WGS)-based analysis (Larman et al., 2012) (Table S6). On the basis of previous functional studies in oncocytoma (Gasparre et al., 2008; Mayr et al., 2008; Simonnet et al., 2003), and because many of our variants represented frameshift substitutions, these mtDNA mutations are thought, in general, to lead to inactivation, rather than activation, of the associated protein.

ETC complex I genes were altered in 18% of cases (n = 11; Figures 1B and 3A; Table S3); the most frequently altered gene was MT-ND5, in six cases (all with >70% heteroplasy), with five of these being histologically classified as eosinophilic ChRCC (p < 0.01, one-sided Fisher’s exact test) and three showing no copy-number abnormalities (p < 0.002). MT-ND5 is essential for the activity of complex I (Chomyn, 2001), which is responsible for the transfer of electrons from NADH to ubiquinone. One ChRCC case had a single base insertion at position 12417 that changes the length of an 8-bp homopolymer tract, which has been observed previously in several other cancer types (Larman et al., 2012); another case had an insertion at 12384, at which position a mutation was found elsewhere in oncocytoma and associated with loss of complex I activity (Mayr et al., 2008). Two ChRCC cases each had single-base deletions at position 13230 of MT-ND5, but no other mtDNA
mutations were recurrent in our cases. We also found \( MT-ND5 \)-mutated ChRCC cases to have a distinct gene transcription signature (Figure 4B; Figures S4A and S4B; 719 genes with \( p < 0.001 \) by \( t \) test, \( FDR < 0.05 \)), which was shared by other eosinophilic cases and not limited to genes in regions of recurrent copy-number abnormality (Figure S4C). Genes with high expression in \( MT-ND5 \)-mutated cases were enriched for those associated with mitochondria (43 with Gene Ontology term “mitochondrion;” \( p < 5 \times 10^{-6} \), one-sided Fisher’s exact test), including several with roles in ETC (\( SDHB \), \( NDUFS1 \), \( ATP5F1 \), \( COX10 \), and \( COX11 \); Table S3). Notably, mutations in complex I did not result in expression patterns associated with loss of oxidative phosphorylation (Figure 4C), as might be assumed (Larman et al., 2012), suggesting possible alternative roles for complex I alteration in cancer-associated metabolic activity (Figure S4D). The associations made here, involving mtDNA mutations with mitochondrial abundance and differential gene expression patterns (which may be unique to ChRCC and related cancers), could perhaps suggest either a compensatory role for loss of complex I function or selective pressures operating to promote alternative pathways.

**Whole-Genome Analysis**

WGS for 50 of our 66 ChRCC cases was performed (60x and 30x coverage for paired tumor and normal, respectively). The Meerkat algorithm (Yang et al., 2013) was applied to detect genomic rearrangements, with an average of 16 found per case (range 0–207; Figure S5A), but without involving recurrent gene-gene fusions. By WGS analysis, a subset of ChRCC manifested kataegis (Figure 5A; Figure S5B), a phenomenon involving highly localized substitution mutations (\( C > T \) or \( C > G \)). Consistent with observations in other cancers (Alexandrov et al., 2013; Nik-Zainal et al., 2012), we found that regions of kataegis in ChRCC were in the vicinity of genomic rearrangements (Figure 5A; Figure S5B; average of 150 rearrangements by pter/qter region). Three ChRCC WGS profiles showed particularly strong patterns involving chromosomal region 3p, 5p, 5q, 8q, 13q, or 15q (Figure 5B). A mutation signature consistent with APOBEC cytidine deaminase activity (Alexandrov et al., 2013; Roberts et al., 2013) was significantly enriched in kataegis regions as well as in tightly spaced mutation clusters forming kataegis events (Figures S5A–S5F and Table S7). Although not detectable in ChRCC WES data (Alexandrov et al., 2013), WGS mutation spectra of six ChRCC cases, including the three with strong kataegis patterns, showed statistically significant (albeit moderate) APOBEC-patterned mutagenesis across the entire genome (Figure S5G). APOBEC3B mRNA expression was also elevated in ChRCC compared with normal kidney (Figure S5G).

We compared gene expression profiles between ChRCC cases with and without a strong kataegis pattern (\( n = 3 \) and \( n = 47 \), respectively) and identified 29 differentially expressed genes (FDR < 0.05) including \( TERT \) (\( p < 1 \times 10^{-10} \), \( t \) test, FDR < \( 1 \times 10^{-6} \); Figure 5C). The \( TERT \) gene itself showed a wide range of expression levels across ChRCC, from undetectable to hundreds of units by RNA sequencing. Focusing our
attention on TERT, we sequenced the promoter region for recently identified mutations (C228T and C250T) (Huang et al., 2013); three cases harbored C228T mutations but were associated with only marginal TERT expression levels (average expression ~1 unit). WGS analysis of DNA copy within the TERT region identified some copy-number variation, but not at levels that would account for the extent of deregulated expression. However, multiple cases did show abrupt changes in copy number.

Figure 5. Kataegis and TERT in ChRCC
(A) Examples of a strong kataegis pattern in two ChRCC cases. “Rainfall” plots of mutations by WGS order events by genomic location. Vertical axis denotes genomic distance of each mutation from the previous mutation.
(B) WGS profiles for 50 ChRCC cases, each scored by genomic region (chromosome pter/qter) for kataegis. The 3 ChRCC cases scoring particularly strong are indicated at the bottom. Score for a given region represents a one-sided Fisher’s exact test, for enrichment of C > T or C > G mutations involving intermutation distances below 10 kb (corrected for testing of multiple regions).
(C) A set of 29 differentially expressed genes (FDR < 0.05), including TERT, observed in ChRCC cases with strong kataegis versus other ChRCC.
(D) Copy variation and DNA breakpoint analysis identifying genomic rearrangements involving the promoter region of TERT for the 50 ChRCC cases (case ordering the same for B, C, and D). The 6 cases harboring rearrangements involving TERT are indicated (pink triangles).
(E) TERT expression levels in the ChRCC cases with TERT promoter SV, in the ChRCC cases with TERT promoter mutation (SNV), and in the remaining cases, as well as in normal kidney samples; p values by two-sided t test on log-transformed data. Box plots represent 5%, 25%, median, 75%, and 95%.

See also Figure S5 and Table S7.
at points that fell within the region 10 kb upstream of the \textit{TERT} transcription start site (Figure 5D). This observation suggested the existence of structural breakpoints, leading us to reexamine our Meerkat-generated results with greater scrutiny.

Subsequent WGS analysis identified genomic rearrangements involving the \textit{TERT} promoter region, leading to breakpoints within the region in six out of 50 ChRCC cases (Figure 5D and Table 2); these cases also had the highest levels of \textit{TERT} expression (average > 500 units, \(p < 1 \times 10^{-20}\), t test; Table 2 and Figure 5E), even compared with cases with 228T mutation, and three showed the strongest manifestation of kataegis (\( p = 0.001\), one-sided Fisher’s exact). In five ChRCC cases, the \textit{TERT}-associated rearrangements were intrachromosomal (one involving part of \textit{PDCD6}), while the sixth case involved \textit{NEK5} on chromosome 13. When considering intratumor heterogeneity, in most cases, these variants were estimated to reside in nearly all of the cells (when counting the numbers of concordant versus discordant read pairs), which would indicate that the \textit{TERT}-associated rearrangements represent early events and therefore possible drivers. Of the seven rearrangements identified by WGS, we confirmed six (involving six cases) by PCR, by designing primers that spanned both sides of the breakpoint junction (Figure 6A; Table S8), allowing amplification of DNA spanning the breakpoint region in the tumor sample (Figure 6B; Figure S6); subsequent sequencing of the PCR product independently confirmed the junction in each case (Figure 6C). Although point mutations in the \textit{TERT} promoter, leading to upregulation of \textit{TERT} itself, have been recently reported in cancers such as melanoma (Heidenreich et al., 2014; Huang et al., 2013), our results represent another phenomenon, of recurrent genomic rearrangement breakpoints in the \textit{TERT} promoter being associated with elevated \textit{TERT} expression in cancer. A precise mechanism remains to be elucidated, though, as a result of rearrangement, a number of \textit{cis}-regulatory elements were found to be placed in close proximity to the core promoter of \textit{TERT} (Figure S7).

### DISCUSSION

With this comprehensive molecular survey of ChRCC, we have made several important findings, in particular the observed recurrent genomic structural rearrangements involving the \textit{TERT} promoter region and elevated \textit{TERT} expression, and our results raise intriguing questions regarding cancer, involving the role of mtDNA alterations and the role of the cell of origin. The above key findings were made possible only by our comprehensive approach, whereby, for example, we had no prior hypotheses regarding \textit{TERT} at the outset of our study. Additionally, mtDNA mutations in cancer, particularly those involving \textit{MT-ND5} and complex I, have been hypothesized elsewhere to recapitulate the Warburg effect (Larman et al., 2012), though the corresponding expression and histological patterns observed in our data were consistent with a complex metabolic phenotype rather than simple loss of oxidative phosphorylation. Taken together, our key findings further illustrate the need to survey cancers outside of exome boundaries, for example, by incorporating WGS or mtDNA sequencing as part of an integrative, multiplatform analysis.

Through integration of molecular data from less common cancers, we can learn more about more frequently encountered diseases. Here, for example, our analysis of ChRCC led to additional insights regarding ccRCC. RCC represents a collection of highly distinct tumors arising from different lineages within the nephron, with distinct molecular and genetic features reflecting independent processes of tumorigenesis (Linehan, 2012). Given the complexity of function assigned to an organ such as the kidney, different cancers arising from this organ may not necessarily appear similar to each other (Alexandrov et al., 2013; Cancer Genome Atlas Network, 2012). Our multiplatform analyses clearly confirm that ChRCC is a distinct disease entity from, and shares little cell lineage or genomic characteristics with, ccRCC, further reinforcing the notion that disease-specific therapies are needed for rarer tumors such as ChRCC, rather than simply adopting conventional therapeutic strategies used for ccRCC. Given the clear genetic differences between ChRCC and ccRCC, our results would suggest cell of origin as a key factor in disease determination, observations that could inform future efforts to fractionate the pool of susceptible cells for ChRCC or ccRCC modeling or preventative interventions. In addition, these data will serve as a resource for future explorations of other tumors of kidney origin, such as papillary RCCs, while being broadly relevant as well to the study of other cancers.
as metabolic, genomic structural alterations, and cellular factors that influence the spectrum of genetic events contributing to cancer development are further realized.

The gene expression patterns, increased mitochondrial numbers, and histological patterns associated with ChRCC all indicate an increased importance of a distinct mitochondrial respiration program in this disease. Renal oncocytoma, a benign renal tumor that, like ChRCC, may also arise from the distal nephron, shares several similarities with ChRCC (particularly with its eosinophilic subtype), including abundant, eosinophilic cytoplasm and densely packed mitochondria (Amin et al., 2008; Tickoo et al., 2000). Mitochondrial accumulation in renal oncocytomas has been hypothesized to be a compensatory mechanism for inefficient oxidative phosphorylation (Simonnet et al., 2003), whereby loss of complex I activity may result from somatically acquired homoplasmic mutations in mitochondrial complex I genes (Gasparre et al., 2008; Mayr et al., 2008; Simonnet et al., 2003). However, gene expression in ChRCC would indicate that increased oxidative phosphorylation is maintained in complex I-altered tumors, suggesting a metabolic shift supporting the growth of this tumor, and counter to the Warburg-like phenomenon observed in high-grade, high-stage ccRCC and many other cancers (Cancer Genome Atlas Research Network, 2013), which would appear consistent with previous observations, using metabolic imaging to demonstrate uptake of radiolabeled acetate but not glucose in ChRCC (Ho et al., 2012).

In general, cancer cells derive much of their ATP through oxidative phosphorylation (Ward and Thompson, 2012), and cancer-associated reprogramming of mitochondria and of other metabolic pathways, besides glycolysis and the Warburg effect, has recently received much attention (Currie et al., 2013; Ward et al., 2013).
and Thompson, 2012). Further studies to dissect the precise role of mtDNA alterations in cancer, and mitochondrial activities promoting cancer growth, could shed light on how core metabolic pathways may be altered in ChRCC and other malignant diseases.

Our finding of recurrent DNA rearrangement breakpoints within the TERT promoter region in over 10% of evaluated cases represents a mechanism for increased TERT expression in cancer different from point mutations observed in a wide variety of cancers (Heidenreich et al., 2014; Huang et al., 2013), gene amplification (Weir et al., 2007; Chudnovsky et al., 2005), and germline polymorphisms (Rafnar et al., 2009). TERT is well recognized as having roles in telomere maintenance and DNA repair, in which deregulation of telomerase is a ubiquitous feature of human cancers. The previously observed TERT promoter mutations (C228T and C250T) create de novo E-twenty-six/ternary complex factors binding sites, which have been observed to increase transcriptional activity from the promoter by 2- to 4-fold (Huang et al., 2013). Interestingly, the TERT expression levels of the six cases with independently validated TERT promoter rearrangements were much higher than those cases with C228T promoter mutations, suggesting that these rearrangements might have an even more potent effect on upregulation of the gene. The precise mechanism of how these rearrangements affect expression remains to be elucidated; they could possibly involve rearranged cis-regulatory elements or could allow the core TERT promoter to escape from the native condensed chromatin setting (Zhao et al., 2009).

The observed association of TERT with kataegis is also intriguing. Elsewhere, rearrangement of DNA sequences upstream of TERT has been reported in immortalized, nontumorigenic fibroblasts, leading to activated telomerase in cells surviving the crisis stage of immortalization (Zhao et al., 2009), which involves chromosomal instability and rearrangements due to loss of telomere capping activity; in the setting of human cancer, this would suggest that TERT-associated rearrangements would be involved in many cases at an early stage in tumorigenesis.

Future applications of the information presented here will include comparative analysis with other cancer types, for the possible existence elsewhere of structural rearrangements involving promoters for TERT or for other key drivers. As a resource with a large set of whole-genome sequences, integrated with a broad array of high-quality platform data sets, other relationships between genomic structural alterations and transcriptional components, including noncoding RNAs, remain to be uncovered. As our data represent single biopsies, future studies might focus on heterogeneity between biopsies from the same tumor (Gerlinger et al., 2012); additionally, subclonal analysis may shed light on early versus late somatic events in ChRCC tumorigenesis. Our study also revealed that divergent approaches for uncovering mtDNA mutations (long-range PCR [LR-PCR] versus WGS [Larman et al., 2012]) are highly complementary to each other, allowing WGS data from other cancers to be similarly mined for mtDNA mutations, with the additional step of combining these data with those of other platforms, in order to better understand the role of the mitochondria in cancer. Finally, the underlying data sets presented here represent part of an interlocking toolset, which can be combined with those of other cancers (Cancer Genome Atlas Research Network et al., 2013), for further discovery of driver alterations, both within and beyond the exome.

**EXPERIMENTAL PROCEDURES**

**Patient and Sample Characteristics**

With informed consent, biospecimens were collected from newly diagnosed patients with ChRCC undergoing surgical resection and who had received no prior treatment for their disease. Samples were obtained with approval from institutional review boards at Brigham and Women’s Hospital, Memorial Sloan-Kettering Cancer Center, the National Cancer Institute, and The University of Texas MD Anderson Cancer Center. Using a co-isolation protocol, DNA and RNA were purified. Details of sample preparation are described in Supplemental Experimental Procedures.

**Data Generation**

In total, 66 ChRCC cases were assayed on at least one molecular profiling platform (Table 1), which platforms included (1) RNA sequencing, (2) DNA methylation arrays, (3) miRNA sequencing, (4) Affymetrix SNP arrays, (5) WES, (6) WGS, and (7) mtDNA sequencing (using LR-PCR to amplify mtDNA). As described above and in Supplemental Experimental Procedures, both single-platform analyses and integrated cross-platform analyses were performed. Sequence files are available from CTGenHub (https://tcga-data.nci.nih.gov/tcga/).

**WGS and WES Analysis**

Massively parallel sequencing exome capture was performed using NimbleGen (custom designed) VCRome 2.1 (42 MB) according the manufacturer’s instructions. All exome sequencing and WGS was performed on the Illumina HiSeq platform. Basic alignment and initial sequence analysis were carried out using the Mercury analysis pipeline (Reid et al., 2014).

**mtDNA Sequencing Analysis**

mtDNA was isolated from tissue samples using LR-PCR methods. Amplified mtDNA PCR products were constructed into Illumina paired-end libraries, and raw sequence data were preprocessed and aligned using the Mercury pipeline.

**RNA Sequencing Analysis**

Both mRNA and miRNA libraries were separately generated from total RNA and constructed using manufacturer protocols. Sequencing was done on the Illumina HiSeq platform. Read mapping and downstream data analysis were performed as described in Supplemental Experimental Procedures.

**Array Data Analysis**

DNA was hybridized to Affymetrix SNP 6.0 arrays and Illumina Infinium HumanMethylation450 (HM450) BeadChip arrays, according to manufacturer protocols.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and eight tables and can be found with this article online at dx.doi.org/10.1016/j.ccr.2014.07.014.

**CONSORTIA**


AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We wish to thank all patients and families who contributed to this study. This work was supported by the following grants from the NIH: 5U24CA143843 (D.A.W.), U54HG003273 (R.A.G.), SU24CA143868 (M.A. Marra), KL2TR001109 and UL1TR001111 (A.B.S.), 5P50CA101942 (S. Signoret), 5P50CA101942 (T.K.C.), U54 HG003067 (E. Lander); K24CA172355 (W.K.R.), Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research (W.M.L., L.S.S., C.J.R., M.J. Merino), with federal funds from the Frederick National Lab, NIH, under contract HHSN261200800001E (L.S.S.), Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (D.A.G., S.A. Roberts, L.J. Klimczak, D. Fargo), and a training fellowship from the Keck Center for Interdisciplinary Bioscience Training of the Gulf Coast Consortia (Grant No. T15 LM007093 to C.F.D.). Other grant sup- port includes the J. Randall & Kathleen L. MacDonald Kidney Cancer Research Fund, the Tuttle Family Kidney Cancer Research Fund (J.J.H.), the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI13C2096 to W.-Y.P.), and the Korea Institute of Science and Technology Information (K-14-L01-C02-S04 and KSC-2013-C3-037) for (super)computing resources including technical support.

Received: April 24, 2014

Revised: June 29, 2014

Accepted: July 17, 2014

Published: August 21, 2014

REFERENCES


Brunelli, M., Eble, J.N., Zhang, S., Martignoni, G., Delahunt, B., and Cheng, L. (2005). Eosinophilic and classic chromophobe renal cell carcinomas have similar frequent losses of multiple chromosomes from among chromosomes 1, 2, 6, 10, and 17, and this pattern of genetic abnormality is not present in renal oncocytoma. Mod. Pathol. 18, 161–169.


