Decreased Mitochondrial DNA Content Drives OXPHOS in Chromophobe Renal Cell Carcinoma

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ABSTRACT

Chromophobe renal cell carcinoma (chRCC) and renal oncocytoma are closely related, rare kidney tumors. Mutations in complex I (CI)-encoding genes play an important role in dysfunction of the oxidative phosphorylation (OXPHOS) system in renal oncocytoma, but are less frequently observed in chRCC. As such, the relevance of OXPHOS status and role of CI mutations in chRCC remain unknown. To address this issue, we performed proteome and metabolome profiling as well as mitochondrial whole-exome sequencing to detect mitochondrial alterations in chRCC tissue specimens. Multimomic analysis revealed downregulation of electron transport chain (ETC) components in chRCC that differed from the expression profile in renal oncocytoma. A decrease in mitochondrial (mt)DNA content, rather than CI mutations, was the main cause for reduced OXPHOS in chRCC. There was a negative correlation between protein and transcript levels of nuclear DNA- but not mtDNA-encoded ETC complex subunits in chRCC. In addition, the reactive oxygen species scavenger glutathione (GSH) was upregulated in chRCC due to decreased expression of proteins involved in GSH degradation. These results demonstrate that distinct mechanisms of OXPHOS exist in chRCC and renal oncocytoma and that expression levels of ETC complex subunits can serve as a diagnostic marker for this rare malignancy.

Significance: These findings establish potential diagnostic markers to distinguish malignant chRCC from its highly similar but benign counterpart, renal oncocytoma.

Introduction

Chromophobe renal cell carcinoma (chRCC) and renal oncocytoma (RO) originate from intercalated cells of the collecting duct, with each accounting for approximately 5% of all renal neoplasms (1, 2). The World Health Organization (WHO) chRCC classification guidelines define a classic and an eosinophilic variant (1). Classic chRCC (about 75%–80% of chRCC) consists of large cells with reticular cytoplasm and prominent cell membranes (pale cells) that are characterized by numerous cytoplasmic microvesicles, which is probably related to defective mitochondrial development, whereas mitochondria are abundant in the eosinophilic phenotype (3–6). The characteristic genetic feature of chRCC is monosomy of chromosomes 1, 2, 6, 10, 13, 17, and often 21 (7, 13, 17). Although the typical mutations in tumor protein 53 (TP53; 52%) and PTEN (9%) genes have been reported (7, 10, 11), chRCC has a low mutational burden overall; there are no clear driver mutations in >50% of chRCC cases (10, 11). This is in contrast to clear cell (cc)RCC, in which the frequency of the Von Hippel-Lindau (VHL) gene mutation is >80% (12).

RO is classified as a benign renal epithelial neoplasm (13, 14) that is usually treatable by nephrectomy. The main molecular hallmark of RO is the marked reduction or complete loss of complex I (CI) enzyme activity in the electron transport chain (ETC; refs. 15, 16). This is primarily caused by mutations in mitochondrial (mt)DNA and especially, but not exclusively, in genes encoding CI subunits (15, 17). Most of the mtDNA mutations are well above the threshold for a pathogenic phenotypic effect resulting from high heteroplasmic loads (15, 17) and inhibit tumor growth through adverse effects on respiratory complex assembly (18, 19). Thus, mtDNA mutations are thought to be the main cause of the indolent, low-proliferative, noninvasive behavior of RO and are driving the cancer phenotype (17, 20, 21).

chRCC is considered as the malignant counterpart to RO (21) with similar origin, gene expression patterns, and mitochondrial pathology, making them difficult to distinguish histologically (2, 10, 21). The two tumor types have distinct mtDNA mutation profiles (18, 19). In contrast to RO, only 13% of chRCC cases harbor CI mutations, with a heteroplasmic rate >50% (10). Transcriptome analyses have shown that chRCC cases with mutations in CI subunit-encoding genes did not have RNA expression patterns associated with loss of oxidative phosphorylation (OXPHOS; refs. 10, 22), contrary to expectation (23). This suggests possible alternative roles for CI mutations in OXPHOS or, as-yet unknown, compensatory mechanisms leading to increased transcript levels of OXPHOS-related factors. It was recently reported that chRCC and RO have elevated levels of GSH (24–28), which is a novel hallmark of all RCCs and a potential therapeutic target (29).

Enhanced GSH biosynthesis in RO is presumed to be an adaptive event resulting from loss-of-function mutations in CI subunits (24, 25). However, given the low frequency of CI mutations in chRCC (10), it is likely that the elevated GSH level is the result of an alternative mechanism.
Here we demonstrate that protein levels of ETC complex subunits were decreased, whereas global mitochondrial mass was unchanged in chRCC compared with healthy kidney tissue. This is in contrast to RO, in which there was a general increase in mitochondrial mass but not in CI subunit expression. There was a negative correlation between protein and mRNA levels of ETC complex subunits in chRCC, and a reduction in mtDNA content rather than CI mutations was found to be responsible for the decrease in OXPHOS. Interestingly, GSH levels increased with mtDNA depletion. These features may serve as markers for distinguishing between these two closely related tumor types.

Materials and Methods

Sample acquisition and verification

Nine pairs of chRCC and adjacent healthy kidney tissue derived from nephrectomies at the Charité – Universitätsmedizin Berlin were collected in liquid nitrogen within 30 minutes after surgery and preserved at −80°C. The clinical characteristics of the tumors are reported in Supplementary Table S1. From the collected tissue samples, histologic sections were stained with hematoxylin and eosin. The diagnosis of chRCC and the corresponding matched tumor-free kidney tissue was done according to the WHO classification criteria. Only cases with a clear diagnosis of chRCC were considered for the study. The typical genetic feature of chRCC, the monosomy of chromosomes 1, 2, 6, 10, 13, 17, and 21, was confirmed in all cases by analyzing the chromosome copy number variation based on the whole-exome sequencing data (Supplementary Fig. S1). The study was approved by the institutional Ethics Committee (no. EA1/134/12) and was carried out in accordance with the Declaration of Helsinki. All participants gave written informed consent.

Proteomic analysis

The tissue samples for proteomics were processed with iST 96X kits following the manufacturer’s protocol (iST sample preparation kit 96X, PreOomics). Briefly, tissues were homogenized under denaturing conditions with a FastPrep (three times for 60 seconds, 6.5 m s⁻¹), followed by boiling at 95°C for 10 minutes. The lysates containing 40 μg protein were then digested by trypsin and Lys-C protease mixture at 37°C overnight. Subsequently, the peptides were purified with the cartridge and each sample was further separated using three fractions according to (30). A total of 10 μg of each fraction was analyzed by LC-MS for proteome profiling. All fractions were allocated to the corresponding replicate and analyzed jointly by the software tool MaxQuant (31).

LC/MS-MS was carried out by nanoflow reverse-phase LC (Dionex Ultimate 3000, Thermo Fisher Scientific) coupled online to a Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific). Briefly, the LC separation was performed using a PicoFrit analytic column (75 μm ID × 55 cm long, 15 μm Tip ID; New Objectives) in-house packed with 3-μm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Peptides were eluted using a gradient from 3.8% to 40% solvent B in solvent A over 120 minutes at 266 nL per minute flow rate. Solvent A was 0.1% formic acid and solvent B was 79.9% acetonitrile, 20% H₂O, 0.1% formic acid. Nanoelectrospray was generated by applying 3.5 kV. A cycle of one full Fourier transformation scan mass spectrum (300–1,750 m/z, resolution of 60,000 at m/z 200, AGC target 10⁶) was followed by 16 data-dependent MS/MS scans (resolution of 30,000, AGC target 5e⁵) with a normalized collision energy of 27 eV. To avoid repeated sequencing of the same peptides, a dynamic exclusion window of 30 seconds was used. In addition, only peptide charge states between two to eight were sequenced.

Raw MS data were processed with MaxQuant software (v1.6.0.1, ref. 31) and searched against the human proteome database UniProtKB with 70,941 entries, released in January, 2017. Parameters of MaxQuant database searching were: A false discovery rate (FDR) of 0.01 for proteins and peptides, a minimum peptide length of 7 amino acids, a mass tolerance of 4.5 ppm for precursor and 20 ppm for fragment ions were required. A maximum of two missed cleavages was allowed for the tryptic digest. Cysteine carbamidomethylation was set as a fixed modification, while N-terminal acetylation and methionine oxidation were set as variable modifications. MaxQuant processed output files can be found in Supplementary Table S2, showing peptide and protein identification, accession numbers, percent sequence coverage of the protein, q values, and LFQ intensities. Contaminants, as well as proteins that were only identified by site modifications and proteins derived from the reversed part of the decoy database, were strictly excluded from further analysis.

Metabolite profiling

About 30 mg of nine unrelated and N₂ shock frozen chRCC and corresponding healthy kidney tissues were used for metabolite profiling. Metabolite extraction and tandem LC-MS/MS measurements were done as previously reported by us (24). In brief, methyl-tetrt-butyl ester (MTBE), methanol, ammonium acetate, and water were used for metabolite extraction. The subsequent separation was performed on an LC instrument (1290 series UHPLC; Agilent), online coupled to a triple quadrupole hybrid ion trap mass spectrometer QTrap 6500 (Sciex, Foster City, CA), as reported previously (32). Transition settings for multiple reaction monitoring (MRM) are provided in Supplementary Table S3.

The metabolite identification was based on three levels: (i) the correct retention time, (ii) up to three MRMs, and (iii) a matching MRM ion ratio of tuned pure metabolites as a reference (32). Relative quantification was performed using MultiQuant software v.2.1.1 (Sciex). The integration setting was a peak splitting factor of 2 and all peaks were reviewed manually. Only the average peak area of the first transition was used for calculations. Normalization was done according to used amounts of tissues and subsequently by internal standards, as indicated in Supplementary Table S3.

Determination of free and total GSH in plasma and urine

The GSH level in plasma- (6 RO, 6 ccRCC, 12 pRCC, 6 chRCC, and 6 healthy) and urine specimens (8 RO, 20 ccRCC, 19 pRCC, 7 chRCC, and 20 healthy) was investigated to see if it can be used as a non-invasive metabolic marker. Free and total GSH were measured using a GSH fluorescent detection kit (catalog no. EIAGSHF) according to the manufacturer’s protocol (Invitrogen).

Enzyme activity measurement

Sample preparation to spectrophotometrically assay enzyme activity was done as reported previously (32). In brief, approximately 5 mg of the tumor and healthy kidney tissues were homogenized and centrifuged at 600 g at 4°C for 10 min. The protein concentrations of supernatants were further determined with a BCA assay (Thermo Fisher, Germany). For complex I, II, and V, 4 μg protein of each sample were used for the enzymatic activity measurement; for complex III, and IV, 2 μg protein was used. Rotenone (10 μmol/L), malonic acid (5 mmol/L), antimycin A (5 μg/mL), potassium cyanide (500 μmol/L), and oligomycin (5 μg/mL) served as specific inhibitors for complex I to V, respectively.
Whole exome sequencing and mitochondrial bioinformatics analysis

DNA was isolated from the remaining pellets after metabolite extraction and was sequenced using a library purification kit according to the manufacturer’s protocol (QiAamp DNA Mini Kit for Tissues, QIAGEN, Hilden, Germany). In brief, the pellets were lysed by proteinase K and the RNA was removed by RNase, the RNA-free genomic DNA was then purified and eluted on QiAamp Mini spin columns for library preparation. The library preparation was performed according to Agilent’s SureSelect protocol (SureSelectXT Human All Exon V5, protocol version B4 August 2015) for Illumina paired-end sequencing, as reported previously (24). The fastq files were used as input for the MToolBox pipeline (33), to extract mitochondrial DNA sequences and quantitate each variant allele heteroplasmy, as done previously (24). Copy number variation (CNV) was inferred and visualized with the CNVkit (34).

Analysis of The Cancer Genome Atlas RNA-seq data

The Cancer Genome Atlas (TCGA; ID: KICH) RNA-seq data were obtained from UCSC Xena (ref. 35; https://xenabrowser.net/). Accurate transcript quantification of chRCC (n = 66) and controls (n = 25) was based on the RNA-Seq by the Expectation Maximization method (36).

Cell culture

The recently established and chRCC-derived cell line UOK276 (37) was a kind gift from Prof. Marston Linehan (Center for Cancer Research at the NCI, Bethesda, MD). The ccRCC derived cell line 786-O and the normal kidney cell line HK2 were obtained from ATCC. No Mycoplasma testing was performed before the experiments. The three cell lines were cultivated in DMEM (Life Technologies) containing 4.5 g/L glucose, supplemented with 10% FBS (Silantes) and 1% penicillin-streptomycin-neomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO2.

The three types of cells were seeded into 6-well plates and the cell medium was supplemented with 100 ng/mL ethidium bromide (EtBr) and 50 μg/mL uridine to induce mtDNA depletion. On days 0, 2, 5, 8, 13, and 19, the cells were collected and frozen immediately at −80°C for the measurement of GSH, mtDNA, RNA, and protein correlation.

Western blotting

The proteins were extracted from the cells by using the TRizol reagent (Sigma), and then resolved by SDS–PAGE. Separated proteins were transferred to PVDF membranes and incubated with indicated primary antibodies diluted in TBST (20 mmol/L Tris, 150 mmol/L NaCl, and 0.1% Tween 20) supplemented with 5% BSA (Sigma). Primary antibodies were detected with HRP-conjugated secondary antibodies followed by exposure to ECL reagents (Bio-Rad). The used primary antibodies and dilutions are as follows: anti MT-ND5 (Thermo Fisher Scientific, PA5-39777, 1:500); anti NDUF51 (ProteinTech, 12444-1-AP, 1:1,000); anti SDHB (Sigma, HPA002868, 1:500); anti UQCR2 (Sigma, HPA007998, 1:250).

qPCR

Total RNA and the genomic DNA were isolated using the TRizol reagent (Sigma), and the QIAamp DNA micro kit (Qiagen) according to the manufacturers’ instructions, respectively. The quantitative PCR analysis was performed using GoTaq qPCR regents (Promega) mixed with indicated primers in a 7900 Real-Time PCR system (Applied Biosystems). Predesigned primers were synthesized by Eurofins Genomics according to the following transcriptions: MT-ND5 (5′-CCGGAAG-CCTATTCGAGGA, 5′-ACAGCGAGGCTTGAGTGT), NDUF51 (5′-TGGTGAAGCCTGTAGTCG, 5′-TGCTCTCTACACACCAG), SDHB (5′-GACGGCTCAGAGTTCAT, 5′-TGATGGTGTTGCCAGGCGTAT), UQCR2 (5′-AACACCGATTGCGCTGTGCC, 5′-TCCCCTGGGGTCCACACT), SLC1A4 (5′-AGCACGCAACTTCCAGTGTG, 5′-GTCGTAGTACGACGCCCA), SDHB (5′-GACGGCTCAGAGTTCAT, 5′-TGATGGTGTTGCCAGGCGTAT), UQCR2 (5′-AACACCGATTGCGCTGTGCC, 5′-TCCCCTGGGGTCCACACT), SLC1A4 (5′-AGCACGCAACTTCCAGTGTG, 5′-GTCGTAGTACGACGCCCA).

Experimental design and statistical rationale and pathway analyses

For proteome and metabolome datasets, a two-sample t test was performed. Multiple test correction was done by Benjamini–Hochberg with an FDR of 0.05 by using Perseus (v1.0.0.2; ref. 38). Significantly regulated proteins and metabolites were marked by a plus sign in the corresponding Supplementary Tables S2 and S3. The Mann–Whitney U test was used to determine whether GSH levels from independent urine and plasma samples have the same distribution; the P value significance cutoff was ≤0.01.

For comprehensive proteome data analyses, gene set enrichment analysis (GSEA, v3.0; ref. 39) was applied to see whether an a priori defined set of proteins shows statistically significant, concordant differences between chRCC and kidney tissues. Only proteins with valid values in at least six of nine samples in at least one group with replacing missing values from the normal distribution for the other group were used (Supplementary Table S2). GSEA default settings were applied, except that the minimum size exclusion was set to 10 and KEGG v6.2 was used as a gene set database. The cutoff for significantly regulated pathways was set to P ≤ 0.01 and FDR ≤ 0.05.

Data availability

The datasets generated in the current study are available as supplementary files and in the following repositories:

- WES files can be accessed via https://www.ncbi.nlm.nih.gov/sra with the accession number: PRJNA413158. Proteomics raw data have been deposited to the ProteomeXchange Consortium via the Pride partner repository (40) with the dataset identifier PXD019123. Metabolomics data have been deposited in the publically available repository PeptideAtlas with the identifier PASS01250 and can be downloaded via http://www.peptideatlas.org/PASS/PASS01250.

Results

Proteomic analysis reveals OXPHOS dysregulation in chRCC

The loss of CI and compensatory increases in the protein levels and enzymatic activity of the other ETC complexes are the primary molecular hallmarks of RO (15, 16, 24, 25). To determine whether mitochondrial dysfunction is also present in chRCC, we carried out a proteomic analysis of 9 chRCC and adjacent normal kidney tissue samples (Supplementary Table S1). There was a general decrease in the expression of ETC complex subunits in chRCC relative to normal kidney tissue that predominantly affected CI (Fig. 1A), as determined with the t test with Benjamini–Hochberg correction [false discovery rate (FDR) < 0.05; Supplementary Table S2]. The average fold change (chRCC vs. kidney) was −1.79 for CI, −1.05 for CII, −0.91 for CIV, and −0.57 for CV, with no change observed for CIII (Fig. 1A). A
Figure 1.
Differential regulation of ETC complexes in chRCC and RO. **A**, Comparison of protein abundance ratios (log2) of ETC complex subunits between chRCC (**A**) and RO (**B**) tissue vs. healthy kidney tissue samples. The 5 ETC complexes—including all quantified subunits and assembly factors—and corresponding log2-fold changes are shown. The color gradient reflects low (blue) or high (red) abundance of the protein in the tumor. The abundance of CI subunits was decreased in chRCC and RO, whereas the levels of all other ETC complexes were increased in RO while generally showing low abundance in chRCC. Assembly factors (bold type) that are not part of the final complex were upregulated in both tumor types. *, FDR ≤ 0.05. **C and D**, Comparison of the density of mitochondrial and nonmitochondrial proteins vs. log2-fold change of proteins between RO and healthy kidney tissue (**C**) and between chRCC and healthy kidney tissue (**D**). Mitochondrial proteins are shown in red (Human Mito Carta, 1158 entries) and nonmitochondrial proteins are shown in blue.
comparison with RO proteome data (24) revealed that only CI subunits were downregulated in both tumors, whereas protein levels of the components of all other complexes were increased in RO and decreased in chRCC relative to normal tissue (Fig. 1A and B). It is worth noting that increased mitochondrial mass, a histologic hallmark of RO, was consistently observed by analysis of the proteome data, with mitochondrial proteins showing an obvious shift toward upregulation (Fig. 1C). This confirmed the strong reduction or complete loss of CI in RO (15, 24), as the increased mitochondrial mass would be expected to reflect an increase in all mitochondrial proteins. However, allocation analysis of the chRCC proteome did not reveal any significant shifts in mitochondrial proteins (Fig. 1D), indicating that the decrease in OXPHOS in chRCC relative to normal kidney tissue does not result from a change in the amount of mitochondria.

A comparative proteome analysis of chRCC and RO revealed a marked reduction of CI in both tumor types, whereas other ETC complexes showed opposite trends in RO versus chRCC (upregulated and downregulated, respectively), suggesting that differences in OXPHOS are a potential marker for distinguishing between the two types of tumor.

**chRCC exhibits a discrepancy between mRNA and protein levels of ETC complex subunits**

In contrast to the decreased abundance of ETC complex proteins observed by proteome profiling, a transcriptome analysis of chRCC (Kidney Chromophobe dataset from TCGA) indicated that genes encoding ETC components were highly expressed (10, 22). GSEA (39) of all quantified proteins showed a negative enrichment score (−0.36) for the Kyoto Encyclopedia of Genes and Genomes pathway “oxidative phosphorylation” (Fig. 2A) but a positive score (0.47) for the transcriptome data (Fig. 2B). We therefore performed a correlation analysis of proteome and transcriptome data and found a high overall correlation between protein and mRNA levels (r = 0.671) similar to the value for all compounds of mitochondria (r = 0.712; Fig. 2C). However, the protein abundance of individual ETC complex subunits showed a low correlation with the corresponding transcript (r = 0.196).

To clarify the biological significance of this inverse and ETC-specific regulation of the proteome and transcriptome, we compared the enzyme activity of all ETC complexes between chRCC and adjacent healthy kidney tissue samples and found a significant reduction in the activities of CI, CII, CIV, and CV, but no change in that of CIII in chRCC (Fig. 2D–H), which was consistent with the observed trend in protein levels (Fig. 1A).

**GSH level is increased, whereas expression of GSH-degrading enzymes is decreased in chRCC**

Reactive oxygen species (ROS) are produced by the ETC, primarily by CI and CIII (41, 42). The levels of GSH, the major intracellular ROS scavenger, as well as its related metabolites are elevated in RO as an adaptive response to CI deficiency (24, 25). Here we found that the top three metabolites with higher levels in chRCC compared with normal kidney tissue were all related to GSH metabolism, that is, GSH (115-fold), glutathione disulfide (GSSG, 54-fold), and γ-glutamylcysteine (15-fold; Fig. 3A–C, Supplementary Table S3). GSH/GSSG ratio, an indicator of cellular oxidative stress status (43), was increased in all chRCC samples except the Case 4 sample (Fig. 3D), implying a general reduction in oxidative stress in chRCC resulting from the ≥100-fold higher GSH and GSSG levels compared with a normal kidney. Contrary with the increased levels of GSH-related metabolites, the GSH metabolism pathway was under-represented (P ≤ 0.005) in the chRCC proteome GSEA (Supplementary Table S2). This was mainly due to downregulation (42-fold lower on average) of GSH-degrading and -conjugating enzymes such as γ-glutamyltransferase (GGT)3, GGT5, glutathione S-transferase mu (GSTM2, GSTM3, GST alpha 1 (GSTA1), and aminopeptidase N (ANPEP), as well as GSH peroxidase (GPX3), which catalyzes the reduction of hydrogen peroxide (Fig. 3E). However, expression of glutathione synthetase and glutamate-cysteine ligase (GSS and GCLC, 2 key enzymes involved in GSH synthesis) and the other GSH-related enzymes, GPX1 and GPX4, the GSH S-transferases GST omega 1 (GSTO1) and GST pi 1 (GSTP1), and GSH reductase (GSR) were not significantly altered (Fig. 3E). Thus, the elevated levels of GSH-related metabolites in chRCC are attributable to a decreased abundance of proteins involved in GSH degradation, and not to an increase in GSH synthesis.

We analyzed free and total GSH levels in the plasma and urine of ccRCC, papillary (p)RCC, chRCC, and RO patients vs. controls to determine whether GSH can serve as a noninvasive diagnostic marker. The results showed that free (Supplementary Fig. S2A and S2B) and total (Supplementary Fig. S2C and S2D) GSH levels were similar across tumor types. Thus, urine or plasma GSH levels are not a useful metabolic marker for distinguishing between renal tumor types and healthy individuals.

**Decreased mtDNA content and mutations lead to OXPHOS dysfunction in chRCC**

To investigate whether mtDNA mutations are the main cause of OXPHOS dysfunction in chRCC, we examined assembled mitochondrial whole-exome sequencing (WES) reads to identify somatic and germline mtDNA mutations in chRCC by pairwise comparisons between tumor and healthy tissues. We found adequate coverage (>99.9%) and quality for reliable mtDNA reconstruction and variant calling (Supplementary Table S4). Neither the common deletion (mtDNA<2477), a 4977 bp deletion of mtDNA that specifically disrupts CI, CIV, and CV on the ETC, which causes a wide spectrum of clinical manifestations (44) and is suspected to be associated with carcinogenesis (45), nor any other mtDNA deletions were detected in our chRCC cohort. Five somatic nonsynonymous events were identified with a high disease score (>0.7) that are potentially pathogenic (Fig. 4A; Supplementary Table S4): 4 involved CI genes from 4 cases and 1 involved a CIV gene. The chRCC specimen for case 4—the only sample with a GSH/GSSG ratio lower than that of adjacent healthy tissue—harbored a somatic mutation in the mitochondria-encoded NADH-ubiquinone oxidoreductase chain 5 protein gene (MT-ND5); >60% heteroplasmy. The very low rate of heteroplasmy for the other events, that is, 2 events <30%, 2 at approximately 5%, and no nonsynonymous CI mutations in the remaining chRCC cases (Fig. 4A; Supplementary Table S4), suggests that mechanisms other than loss-of-function mutations in CI lead to OXPHOS dysfunction and increase GSH levels in some chRCC cases.

p53 cells are devoid of mtDNA and show drastic reduction of all ETC proteins (46). As a lower mtDNA copy number has been reported in chRCC (47), we speculated that the decreased levels of ETC complex subunits could be explained by a fewer number of copies of mtDNA. To evaluate mtDNA copy number in chRCC, we compared mtDNA reads between chRCC and healthy kidney tissue, as read depth in a specific region of the genome is roughly proportional to DNA copy number in that region. The mtDNA read depth was decreased 3-fold in

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Figure 2.
Comparative analysis of RNA and protein abundance between chRCC and healthy kidney tissue. A and B, GSEA revealed that ETC complex components were downregulated at the protein level (this study; A) and upregulated at the transcript level (TCGA data; B). C, Global transcript versus protein levels are shown as blue dots, proteins localized to mitochondria as red triangles, and specific factors involved in the ETC as green squares, indicating the discrepancy between transcript and protein levels exclusively for the ETC. RNA data were retrieved from TCGA. Comparison of enzyme activity (nmol/min/mg protein, n = 9) of ETC complexes between chRCC and healthy kidney tissue, including CI (D), CII (E), CIII (F), CIV (G), and CV (H). *P < 0.05; **P < 0.01; ***P < 0.001 (paired t test); n.s., not significant.
Figure 3.
GSH levels are elevated in chRCC. Relative abundance of metabolites involved in GSH metabolism is shown for chRCC and healthy kidney tissue samples. **A**, Levels of reduced GSH (A), oxidized GSH (B), and gamma-glutamylcysteine (C) are shown. **D**, GSH/GSSG ratio calculated on the basis of relative signal intensity. **E**, Heatmap of quantified proteins involved in GSH metabolism in chRCC. The color gradient represents a low (blue) or high (red) log2-fold change in chRCC versus normal kidney tissue. ***, P < 0.001; ***, P < 0.001 (two-tailed Student t test). A.U., arbitrary units.

Figure 4.
Identified somatic mtDNA mutations and mtDNA copy number in chRCC. **A**, Somatic mtDNA mutations with a high disease score (>0.7) were detected in 5 tumors; only the MT-ND5 mutation in case 4 had a significant heteroplasmy rate. **B**, Decreased mtDNA read depth indicating a lower mtDNA copy number in chRCC compared with normal kidney tissue (n = 9). *, P < 0.05 (paired t test).
chRCC relative to normal tissue (Fig. 4B), indicating a lower mtDNA content.

Low mtDNA content causes downregulation of ETC complex subunits and elevation of GSH levels

To investigate the influence of mtDNA content on protein and transcript levels of ETC complex subunits and GSH levels, UOK276 (a chRCC cell line; ref. 37), was chronically exposed to a low concentration of ethidium bromide (EtBr) for 19 days to deplete mtDNA (48). Samples collected on days 0, 2, 5, 8, 13, and 19 for analysis showed that mtDNA content decreased within 5 days and reached $r^0$ status (complete mtDNA depletion) after 13 days (Fig. 5A). The protein levels of 4 ETC complex subunits, including those encoded by mtDNA [MT-ND5 (CI)] and nuclear DNA (NADH:ubiquinone oxidoreductase core subunit [NDUFS]1 [CI], succinate dehydrogenase complex iron sulfur subunit [SDH]B [CII], and ubiquinol-cytochrome C reductase core protein [UQCRC]2 [CIII]), were analyzed by Western blotting. All subunits showed a time-dependent decrease in expression and were almost undetectable after 13 days (Fig. 5B), implying a direct link between mtDNA depletion and a reduction in ETC proteins.

We next performed metabolome profiling to determine whether GSH levels correlated with mtDNA depletion in $r^0$ cells and found that they increased during the process of mtDNA depletion, reaching a plateau on day 13 (Fig. 5C). Interestingly, there were 2 distinct mRNA expression profiles for the 4 ETC complex subunits: nuclear DNA-encoded transcripts decreased until day 8 (NDUFS1) or day 13 (SDHB and UQCRC2), before increasing (Fig. 5D). The mRNA levels were mostly unrelated to those of the corresponding protein (Fig. 5B). A notable exception was the transcript level of the mtDNA-encoded MT-ND5, which decreased after just 2 days and did not recover (Fig. 5D), closely mirroring protein expression (Fig. 5B). The same EtBr experiments were performed in the ccRCC cell line 786-O (harboring the Von Hippel-Lindau gene mutation) and normal human kidney cell line (HK-2) to see whether the decrease of mtDNA content and OXPHOS dysfunction is chRCC specific. Similar results were found in these two cell lines compared with that in UOK276 cells (Supplementary Fig. S3A–S3D). Taken together, these results indicate that the decrease in mtDNA content is the main cause of OXPHOS dysfunction in chRCC and leads to an elevation in GSH levels.

Discussion

Although chRCC was identified 30 years ago as a type of kidney cancer (49), it is not well understood because of its rarity. Two recent studies comprising around 100 cases investigated the genetic causes of chRCC, but failed to identify obvious driver mutations in >50% of cases (10, 22). In contrast, mutations in CI genes and mitochondrial abnormalities are frequently observed in RO. To investigate the characteristics of mitochondria in chRCC and their role in disease progression, we used a combination of proteomic, transcriptomic (TCGA), and metabolomic approaches, and performed mitochondrial WES to elucidate the mtDNA mutation landscape and identify differences in mitochondrial function between chRCC and healthy adjacent kidney tissue. Our results showed that a decrease in mtDNA content, and not mutational load, was the main cause of the observed decreases in the levels and activity of nearly all ETC complex subunits (except for CIII), which increased GSH levels and resulted in a discrepancy between mRNA and protein levels of these subunits.
As in many cancer types, glycolytic metabolism is enhanced in chRCC (27). In company with the downregulation of OXPHOS found in this study, we propose that chRCC features the typical “Warburg effect.” We previously reported that the activity of all mitochondrial ETC enzymes was reduced in ccRCC and pRCC (47). This is in contrast to the benign RO, in which only CI subunits show reduced expression and activity, which are increased for all other complexes (24).

The metabolites showing the greatest increase in chRCC relative to healthy adjacent kidney tissue were those involved in GSH metabolism (GSH, GSSG, and gamma-glutamylcysteine), which is consistent with findings for chRCC (26, 27), pRCC (50), RO (24, 25), and ccRCC (51, 52). As GSH is an ROS scavenger (53), the increased GSH levels and GSH/GSSG ratio in chRCC may be a strategy for the tumor to overcome ROS stress originating from dysregulated OXPHOS. As in RO, the abundance of enzymes involved in GSH synthesis was unchanged in chRCC, whereas the level of enzymes involved in GSH degradation was reduced, resulting in high levels of GSH in tumor cells and a microenvironment with a low oxidative stress burden.

The opposite trends in transcript and protein levels of ETC components observed in our chRCC cohort has also been reported in RO (24). The negative correlation suggests that the pathogenic mechanisms of RCC can only be understood by evaluating different levels of molecular information. For example, the enzymatic activity of ETC complex subunits in chRCC and RO (15) corresponds to the abundance of the proteins and not gene expression. A study of mtDNA-depleted p53 cells showed that the levels of all ETC components were significantly reduced because complex assembly is not possible without the core mtDNA-encoded subunits (46). Thus, the inverse relationship between transcript and protein levels in chRCC was mainly due to reduced mtDNA content. This was confirmed by monitoring mtDNA depletion and the abundance of ETC complex subunits over time in a chRCC cell line. All proteins involved in OXPHOS were decreased in parallel with mtDNA content, but there was a discrepancy between mtDNA- and nuclear DNA-encoded ETC complex subunits: the former almost completely disappeared, whereas the latter remained stable when cells reached p53 status. In addition, GSH levels were negatively correlated with mtDNA content, indicating that defective respiration might cause ROS stress, which is compensated for by an increase in the level of GSH, the main ROS scavenger. Another possibility is that the dysfunctional OXPHOS impairs the turnover of NADH and NADPH and thus keeps reduced GSH in chRCC at a higher level.

The mitochondrial genome in RO and thyroid cancers have a high mutational burden (15, 17, 54). Although we identified multiple potentially pathogenic mtDNA mutations with mostly low heteroplasmy loads (except case 4, which harbored a mutated MT-ND5 gene with >60% heteroplasmy), these low heteroplasmic mtDNA mutation rates are unlikely to influence chRCC phenotype as the cases did not show differential regulation of OXPHOS and shared the same overall proteome profile. Although all chRCC cases had similarly high levels of GSH and related metabolites, case 4 showed a decreased GSH/GSSG ratio in chRCC relative to normal kidney tissue, unlike the other cases. This indicates that oxidative stress was increased by the mutation in the MT-ND5 gene, possibly through increased conversion of GSH to GSSG as excessive ROS are produced by CI deficiency caused by MT-ND5 mutations.

Histopathologic differentiation of benign RO from malignant chRCC remains challenging, even by immunohistochemistry using multiple markers (55). In addition, there are case reports on rare hybrid tumors (56, 57), including oncocyctic content, and the rare genetic disorder Brit-Hogg-Dube syndrome (58), in which RO and chRCC coexist. Microvesicle accumulation in the cytoplasm, which has been attributed to defective mitochondriogenesis, is a key histologic feature of chRCC, but has no diagnostic utility as it is also observed in RO and eosinophilic variants of ccRCC (4). Our data demonstrate that the expression profiles of ETC complex subunits can be used to distinguish these two tumor species. However, we want to point out that the phenotype of the 9 chRCC cases in our cohort are obviously very similar and probably belong to the classic variant, as no mitochondrial mass increase was observed by a case-by-case mitochondrial allocation analysis of the proteome data.

In summary, chRCC is characterized by downregulation of the components of the ETC and increased GSH levels, which are mainly caused by decreased mtDNA content and not CI mutation. Moreover, decreased mtDNA content in chRCC underlies the negative correlation between protein and transcript levels of nuclear DNA, but not mtDNA-encoded ETC complex subunits. These results provide insight into the molecular basis for chRCC pathology as well as markers for distinguishing this rare neoplasm from the closely related RO.

Disclosure of Potential Conflicts of Interest

K. Jung reports personal fees from Foundation of Urologic Research, Berlin, Germany during the conduct of the study. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Y. Xiao: Conceptualization, data curation, formal analysis, validation, visualization, methodology, writing-original draft, writing-review and editing. R. Clima: Formal analysis, visualization. J. Busch: Resources. A. Rabien: Resources, writing-review and editing. E. Kilic: Validation. S.L. Villegas: Resources. B. Timmermann: Resources. M. Attimonielli: Formal analysis. K. Jung: Resources, data curation, writing-review and editing. D. Meierhofer: Conceptualization, supervision, funding acquisition, methodology, writing-original draft, project administration, writing-review and editing.

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