

Insights into novel cellular injury mechanisms by gene expression profiling in nephropathic cystinosis

Poonam Sansanwal · Li Li · Szu-Chuan Hsieh ·
Minnie M. Sarwal

Received: 25 June 2010 / Revised: 18 August 2010 / Accepted: 24 August 2010 / Published online: 24 September 2010
© SSIEM and Springer 2010

Abstract Nephropathic cystinosis is a rare, inherited metabolic disease caused by functional defects of cystinosin associated with mutations in the *CTNS* gene. The mechanisms underlying the phenotypic alterations associated with this disease are not well known. In this study, gene expression profiles in peripheral blood of nephropathic cystinosis patients ($N=7$) were compared with controls ($N=7$) using microarray technology. In unsupervised hierarchical clustering analysis, cystinosis samples co-clustered, and 1,604 genes were significantly differentially expressed between both groups. Gene ontology analysis revealed that differen-

tially expressed genes in cystinosis were enriched in cell organelles such as mitochondria, lysosomes, and endoplasmic reticulum ($p \leq 0.030$). The majority of the differentially regulated genes were involved in oxidative phosphorylation, apoptosis, mitochondrial dysfunction, endoplasmic reticulum stress, antigen processing and presentation, B-cell-receptor signaling, and oxidative stress ($p \leq 0.003$). Validation of selected genes involved in apoptosis and oxidative phosphorylation was performed by quantitative real-time polymerase chain reaction (PCR). Electron microscopy and confocal imaging of cystinotic renal proximal tubular epithelial cells further confirmed anomalies in the cellular organelles and pathways identified by microarray analysis. Further analysis of these genes and pathways may offer critical insights into the clinical spectrum of cystinosis patients and ultimately lead to novel links for targeted therapy.

Communicated by: Jean-Marie Saudubray

Competing interest: None declared.

Poonam Sansanwal and Li Li contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s10545-010-9203-6) contains supplementary material, which is available to authorized users.

P. Sansanwal · L. Li · S.-C. Hsieh · M. M. Sarwal
Department of Pediatrics,
Stanford University School of Medicine,
Stanford, CA, USA

M. M. Sarwal (✉)
Department of Pediatrics,
G306, 300 Pasteur Drive,
Stanford, CA 94304, USA
e-mail: msarwal@stanford.edu

P. Sansanwal (✉)
Department of Pediatrics,
S378, 300 Pasteur Drive,
Stanford, CA 94304, USA
e-mail: poonams@stanford.edu

Abbreviations

FDR	False discovery rate
<i>CTNS</i>	Cystinosin
SAM	Significance analysis of microarray
PAM	Prediction analysis of microarray
LSD	Lysosomal storage disorders
RPTE	Renal proximal tubular epithelial

Introduction

Cystinosis is a metabolic disease characterized biochemically by an abnormally high intracellular content of free cystine in different organs due to a transport defect of cystine in

lysosomes (Cherqui et al. 2001; Kalatzis et al. 2001). In untreated patients, disease progression leads to renal Fanconi syndrome, chronic renal failure, hypothyroidism, photophobia, myopathy and retinal blindness, pancreatic dysfunction, as well as central nervous system (CNS) calcifications and symptomatic deterioration (Gahl et al. 2002). In the United States, cystinosis accounts for approximately 1.4% of children on dialysis and 2% of pediatric kidney transplant patients (NAPRTCS 2008). Whereas measurement of free cystine in purified polymorphonuclear leukocytes provides an accurate method of diagnosing and detecting heterozygous carriers, the molecular basis of disease heterogeneity is not well understood. Mutations in the underlying gene, *CTNS* (encoding cystinosin), have been identified as a cause of nephropathic cystinosis (Town et al. 1998). A large number of genetic variants have been characterized in *CTNS* that lead to variants of infantile nephropathic cystinosis, such as adolescent or ocular cystinosis (Anikster et al. 1999). Nevertheless, poor clinical correlation exists between genotype and phenotype, when relating the different *CTNS* mutations to clinical presentations. However, in vitro studies have shown a correlation between the level of cystine transport and severity of symptoms (Kalatzis et al. 2004). The exact mechanisms by which lysosomal cystine accumulation causes a multisystemic disorder with varying degrees of renal injury remain unknown. Chronic renal failure in murine cystinosis knock-out models has been shown to be dependent on the genetic background of mice and not merely on the cystine accumulation itself, again indicating the variation of the renal disorder in cystinosis (Cherqui et al. 2002; Nevo et al. 2010). In humans, Fanconi syndrome and progressive renal injury occurs despite cystine depletion therapy. The mutational spectrum in *CTNS* does not explain the heterogeneity in symptoms; the large variation in the phenotype of the disease suggests the presence of genetic modifiers.

In this study, we investigated whole-genome expression profiles in peripheral blood samples obtained from cystinotic patients in order to identify the modifier gene (s) and pathways associated with the nephropathic form of cystinosis. In addition, the alterations in gene expression and the pathways identified in cystinosis were validated in blood samples and in cultured renal proximal tubule epithelial (RPTE) cells obtained from patients with nephropathic cystinosis.

Methods

Patient and samples

Fourteen whole-blood samples from 14 unique pediatric patients were included in this study. Seven samples were

obtained from cystinotic pediatric patients with the nephropathic variant but without renal insufficiency (Cys), and seven control samples (Ctr) were collected from matched controls. Patients with nephropathic cystinosis were prescribed Cystagon to target a reduction in the white-blood-cell (WBC) cystine levels to $1 < \text{nmol}$ of half-cystine mg^{-1} protein. This group of patients was prescribed Cystagon at a range of 25–50 $\text{mg}/\text{m}^2/\text{day}$ in four divided doses; at least one patient (cys1) admitted to complete nonadherence with Cystagon, and three others admitted to sporadic nonadherence due to drug side effects. Both groups were matched for age (Cys = 13.7 ± 4.9 years with a range of 7.6–21 years, Ctr = 11.1 ± 5.4 years with range of 5.5–16.5 years; $p=0.36$), gender (Cys = 29% female, Ctr = 43% female; $p=0.58$), and race (Cys = 100% white, Ctr = 86% white; $p=0.3$) and creatinine clearance (Cys 112.8 ± 34.7 $\text{ml}/\text{min}/1.73$ m^2 with range of 72–166 $\text{ml}/\text{min}/1.73$ m^2 , Ctr = 87.9 ± 20.6 $\text{ml}/\text{min}/1.73$ m^2 with range of 63–120 $\text{ml}/\text{min}/1.73$ m^2 ; $p=0.12$) at the sample dates. The blood samples were collected between March 2002 and September 2003. Complete blood count (CBC) test was performed on all blood samples. There was no difference in the total WBC count or the percentage of WBC subtypes between patients and controls that may be confounders in the gene expression analysis. Written informed consent was obtained from all participants, and the study was approved by the institutional review board of Stanford University.

Cells and antibodies

RPTE cell cultures were used; two normal primary RPTE cells (Cambrex Biosciences, East Rutherford, NJ, USA) and eight primary cultures from cystinotic RPTE cells isolated from the urine of patients with nephropathic cystinosis (kindly provided by Dr. William Gahl) (Racusen et al. 1995). The study was controlled by institutional review board approvals from the National Institutes of Health and Stanford University. All RPTE cells were cultured in renal epithelial growth medium and prepared according to the manufacturer's instructions (Cambrex). All cells were passaged with trypsin (0.05%) and cultured in a 95% air/5% oxygen (CO_2) Thermo Forma incubator (Waltham, MA, USA) at 37°C . The cells were used for electron microscopy and confocal microscopy experiments. All experiments were performed between passages two and five. Primary antibodies used were mouse monoclonal anti-human Complex V subunit (ATP5F1) purchased from Mitosciences (Eugene, OR, USA). Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin G (IgG) purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Sample collection and RNA extraction

Whole blood was collected from patients in PAXgene™ Blood RNA Tubes (PreAnalytiX, Qiagen), and total RNA was extracted using the PAXgene Blood RNA Kit (PreAnalytiX, Qiagen). Total RNA concentration was measured by NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and the integrity of the extracted total RNA (tRNA) was assessed with the Agilent 2100 Bioanalyzer using RNA Nano Chips (Agilent Technologies, Santa Clara, CA, USA). Total RNA was stored in -80°C until sample preparation for the microarray experiments and quantitative real-time polymerase chain reaction (qRT-PCR).

Microarray target preparation, hybridization, and gene expression profiling

Complementary DNA (cDNA) microarrays were used in this study, which included 32,740 probes containing either characterized genes or expressed sequence tag (EST) printed on glass slides by the Stanford Functional Genomics Facility (<http://microarray.org/sfgf/>). After reannotation by Array Information Library Universal Navigator (AILUN) (Chen et al. 2007), approximately 21,739 of these probes are characterized and mapped to the most recent National Center for Biotechnical Information (NCBI) gene identifiers. Hybridized microarrays were scanned using GenePix 4000 (Axon Instruments, Union City, CA, USA), and fluorescent images were analyzed with the GenePix Pro software package. Data sets for this study were stored in the Stanford Microarray Database (SMD) and gene lists filtered at retrieval. Defective spots were flagged and removed, generating a data file with 14,131 clones under low-stringency retrieval settings (80% representative data, signal to noise ratio of expression measurements >1.5 , and signal >100 in both channels).

Microarray data analysis

Lowess normalization was performed, and gene expression values were transformed to \log_2 (Cy5/Cy3) net fluorescence intensity ratios for analysis. AILUN (Chen et al. 2007) was used to reannotate all probes on cDNA microarray to characterize to the most recent NCBI Entrez gene identifiers. Two-class unpaired significance analysis of microarray (SAM) (Tusher et al. 2001) was used to determine significant differential gene expression between cystinosis and control groups. A false discovery rate ($\text{FDR} < 1.5\%$) was used to determine the differentially expressed genes between the two classes. Prediction analysis of microarray (Tibshirani et al. 2002) was

performed to classify the two groups based on the leave-one-out cross-validation. Cluster and TreeView programs (Eisen et al. 1998) were used to generate and visualize the hierarchical clusters of the samples, measuring similarity of gene expression and across arrays. Three approaches were used to assess the functional composition of genes assessed as significantly associated with cystinosis phenotype: Ingenuity Pathway Analysis (IPA) was used to identify the functional networks, canonical pathways, and involved functions and diseases; Gene ontology (GO) (<http://www.geneontology.org/>) was used to identify the relevant biological process, cellular component, and molecular functions; and Pathway Express (Khatri et al. 2006) was used to identify genes associated with *Kyoto Encyclopedia of Genes and Genomes* (KEGG) pathway. Either hypergeometric gene enrichment scores or an FDR are reported for each functional category in these tools. All statistical analysis was conducted by SAS 9.1.3. All supplemental figures and tables and raw microarray data are available at <http://sarwal.stanford.edu/CTNS/>.

Quantitative real-time PCR

Candidate genes for validation by qRT-PCR were selected based on their statistical and biological significance. Expression of messenger RNA (mRNA) for selected candidate genes was measured in each blood sample of cystinosis and control by qRT-PCR using TaqMan Gene Expression Assay products (Applied Biosystems, Foster City, CA, USA). For each gene, no fluorescent signal was generated by these assays when genomic DNA was used as a template, which confirms that the assay measured only mRNA. Quantitative PCR was carried out in a 384-well plate in duplicate. The relative amount of RNA expression was calculated using a comparative C_T method. Expression values were normalized to 18S ribosomal RNA and control sample on the plate. Student's *t* test was used to compare PCR expression values. The primer list for all selected genes and their corresponding ABI probe IDs are provided in Electronic Supplementary Material (ESM) Table S3.

Electron microscopy

Cells were harvested gently using Trypsin ethylenediaminetetraacetate (EDTA), washed with phosphate-buffered saline (PBS), fixed at 4°C for 2 h with 2% glutaraldehyde in neutral phosphate buffer, postfixed in osmium tetroxide, and embedded in Epon. Sections were cut at 80 nm, stained with lead citrate and uranyl acetate, and examined under an FEI (Hillsboro, OR, USA) Tecnai10 electron microscope.

Confocal microscopy

For immunostaining of mitochondrial Complex protein, cells were plated on chamber slides, washed twice in PBS, fixed in 4% formalin (30 min), and permeabilized with 0.5% Triton X-100 (30 min). Cells were incubated in blocking buffer [PBS (pH 7.2) and 3% bovine serum albumin (BSA)] for 1 h, then washed twice in PBS followed by incubation with the primary antibody for 2 h at room temperature. FITC-conjugated secondary antibody was used to detect bound primary antibody for 1 h at room temperature. Slides were viewed ($\times 63$, oil) using a Leica SP2 AOBS confocal laser scanning microscope, and the images were analyzed by Leica Confocal software (version 2.5).

Results

Global gene expression profiles are identified with cystinosis patients

Unsupervised clustering of the peripheral blood samples shows almost complete segregation of cystinosis and control samples by phenotype, even with unsupervised filtering of a 7,171 gene list, based on a 90% representation and sufficient variability of each gene across all 14 samples, with at least two out of all samples with log₂ ratios greater than an absolute value of 1.5 (ESM Fig. S1).

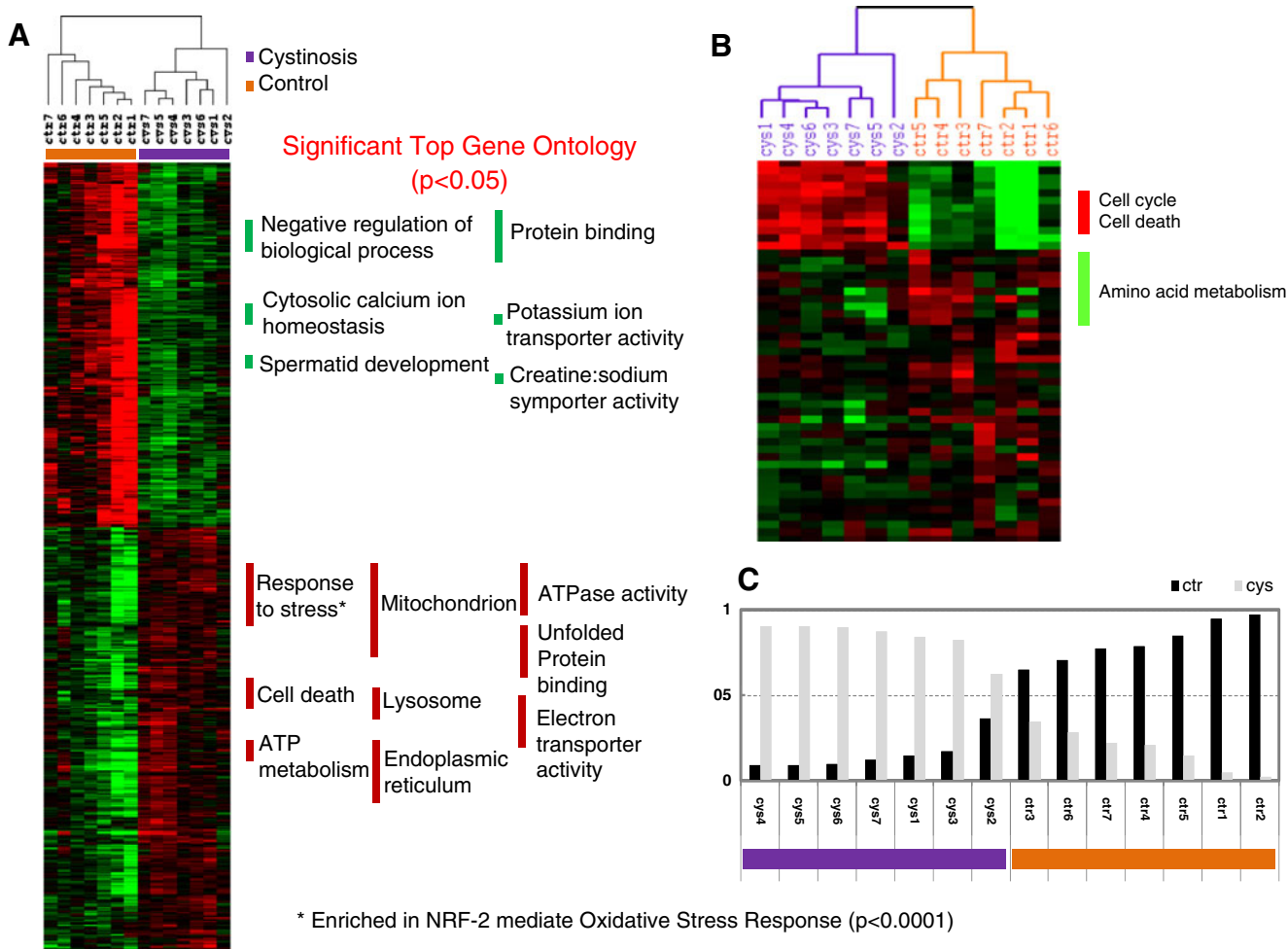


Fig. 1 Minimum gene-set classifiers and significant genes for cystinosis and normal controls: **a** There were 1,604 genes that significantly differentially expressed between cystinosis and controls with false discovery rate (FDR) $< 1.5\%$. Gene ontology with significant biological function (*column 1*), cellular component (*column 2*), and molecular function (*column 3*) for genes involved in up- or downregulated cystinosis are listed next to *heatmap* ($p < 0.05$). **b** The hierarchical

clustering for 14 samples of seven cystinosis and seven control samples is based on the minimum 55-gene set from prediction analysis of microarray (PAM). Genes involved in cell-cycle and cell-death pathways are upregulated in cystinosis samples, whereas genes involved in amino acid metabolism are downregulated. **c** Two-class-prediction probability scores based on PAM are shown for all 14 samples

Table 1 List of genes significantly enriched in lysosome, mitochondria, and endoplasmic reticulum and differentially expressed between cystinosis and control peripheral blood samples

Cellular component	Genes	Fold	False discovery rate	No. genes
Lysosome ($p=0.03$)	<i>ABCA5, NPC1, IFI30, PRCP, GUSB, SCARB2, LAMP2, SMPD1, CTSS</i>	>1.3	<1.2	9
Mitochondrion ($p<0.0001$)	<i>MRPS12, MRPL37, DECR1, SQDRL, VDAC3, SOD2, MTCH1, KARS, COX17, NDUFA2, ATPIF1, UQCRC1, VDAC2, PHB, NDUFA3, RAF1, ETFB, PDHA1, AK2, ATP5J, HAX1, MDH2, HSD17B10, LYRM1, GOT2, UQCRC2, CYCS, DGUOK, MRPS25, C1QBP, SHMT2, MTHFD1, MRPL43, CS, COX6A1, MRP63, ATP5G1, DAP3, UQCR, TIMM10, PNPT1, NDUFA11, MRPS11, NDUFB8, NR3C1, MGST1, ECHS1, ATP5H, PDHA2, COX7A2, SLC25A5, SH3BP5, SSBP1, NDUFA1, ATP5G2, MTHFD2, MRPL11, CPT2, ATP5F1, C14orf159, COX8A, ATP5B</i>	>1.3	<1.2	62
Endoplasmic Reticulum ($p=0.03$)	<i>ALG5, BSCL2, SYNCRIP, VKORC1, MBTPS1, SDCBP, SSR1, SEC61B, HAX1, SSR2, RTN4, SET, EBPL, AB11, ERP29, AHSA1, TMED4, VCP, TRAPPC4, MGST2, UBE2J1, MGST1, GANAB, CANT1, UGCG, HSP90B1, PPIB, EBP, RPN2</i>	>1.4	<1.2	29

Table 2 Canonical pathways significantly overrepresented in the 1,604-probe sets differentially expressed between cystinosis and controls in peripheral blood

Pathway	Up/down in cystinosis	Genes	No. genes
Nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress response ($p=2.95E-06$)	Up	<i>NQO2, MAP3K1, KRAS, FKBP5, MAP2K1, PIK3CD, PRKCB1, PRKCH, DNAJB6, SOD2, TXNRD1, VCP, MGST1, MAP3K5, GSTP1, MGST2, ERP29, PPIB, GSTO1, SOD1, AKR1A1, ATF4, MAPK14, TXN, RAF1, MAF</i>	26
	Down	<i>PIK3R3, DNAJA3, PTPLAD1</i>	3
Oxidative phosphorylation $p=1.37E-04$	Up	<i>ATP5B, NDUFA11, ATP5G1, UQCR, NDUFA3, UQCRC1, NDUFA2, NDUFA1, ATP6V0E1, ATP5G3, ATP6V1E1, COX8A, NDUFB8, COX6A1, COX17, ATP5J, PPA1, ATP5F1, ATP5H, COX7A2, ATP5G2</i>	21
	Down	<i>UQCRC2</i>	1
Apoptosis $p=1.58E-04$	Up	<i>PIK3CD, PRKARIA, IRAK1, TRADD, CAPN2, CYCS, IKBK, CASP4</i>	8
	Down	<i>PIK3RR, PPP3R2, ATM, BCL2L1, AKT2</i>	5
Endoplasmic reticulum stress pathway $p=5.18E-03$	Up	<i>ATF4, EIF2S1, MAP3K5, MBTPS1, XBP1</i>	5
	Down		
Mitochondrial dysfunction $p=3.85E-02$	Up	<i>BACE2, COX17, COX6A1, COX7A2, COX8A, HSD17B10, NDUFA2, NDUFA3, NDUFA11, NDUFB8, PARK7, SOD2, UQCRC1</i>	13
	Down	<i>UQCRC2</i>	1
Antigen processing and presentation $p=0.003$	Up	<i>HLA-DQB1, HSPA8, CTSS, PSME1, HLA-G, HLA-A, IFI30, PSME2</i>	8
	Down	<i>CIITA, HSPA1A</i>	2
B-cell receptor signaling $p=6.89E-07$	Up	<i>ELK1, BCL6, MAP3K1, KRAS, MAP3K2, CAMK2G, MAP2K1, NFATC1, PIK3CD, PRKCB1, VAV1, CDC42, NFKBIE, MAP3K5, NFATC1, PTPN11, RAC1, ATF4, MAPK14, IKBK, RAF1, BCL2A1</i>	21
	Down	<i>CAMK2D, PIK3R3, PPP3R2, BCL2L1, INPP5D, AKT2</i>	6

Significant transcriptional signature is identified for cystinosis

All samples were analyzed by SAM (Tusher et al. 2001), and 1,604 probes with FDR <1.5% were identified. There were 857 genes with upregulated expression, whereas 747 had downregulated expression. Hierarchical clustering showed that distinct cystinosis ($n=7$) and control ($n=7$) samples segregated completely by phenotype (Fig. 1a). Prediction Analysis of Microarray (Tibshirani et al. 2002) perfectly classified the samples with 100% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 100% negative predictive value (NPV), with a minimum gene list of 55 probe identifiers (Fig. 1b, c and ESM Table S1).

Biological relevance of cystinosis signature identification

Gene ontology analysis

GO analysis revealed that upregulated genes were involved in distinct axis for biological processes, such as response to stress ($p=0.016$), adenosine triphosphatase (ATP) metabolism ($p=0.002$), and cell death ($p=0.04$), whereas downregulated

genes were enriched in negative regulation of biological process ($p=0.04$) (Fig. 1a). The gene for cystinosin (*CTNS*), containing the mutation for the cystine lysosomal transporter, was found to be statistically downregulated in cystinosis samples at fold=-2.8 with FDR = 0%. Furthermore, upregulated genes in cystinosis were enriched in lysosome ($p=0.03$), endoplasmic reticulum (ER) ($p=0.03$), and mitochondrion ($p<0.0001$) on cellular component (Fig. 1a and Table 1). Detailed information of all differentially expressed genes localized in the three cellular compartments is given in ESM Table S2. Molecular function of upregulated genes were enriched in electron transporter activity ($p=0.004$), ATPase activity ($p=0.004$), and unfolded protein binding ($p=3.09\times 10^{-5}$), as shown in Fig. 1a.

Pathway analysis

Differently expressed genes in nephropathic cystinosis samples ($n=1,604$ probe sets) were assessed using IPA. Hypergeometric gene-enrichment analysis from IPA shows that the majority of the differentially expressed genes expressed at higher levels in cystinosis showed a strong association with cell-cycle regulation ($p=0.002$) and cell death ($p=0.002$), whereas amino-acid-metabolism-related

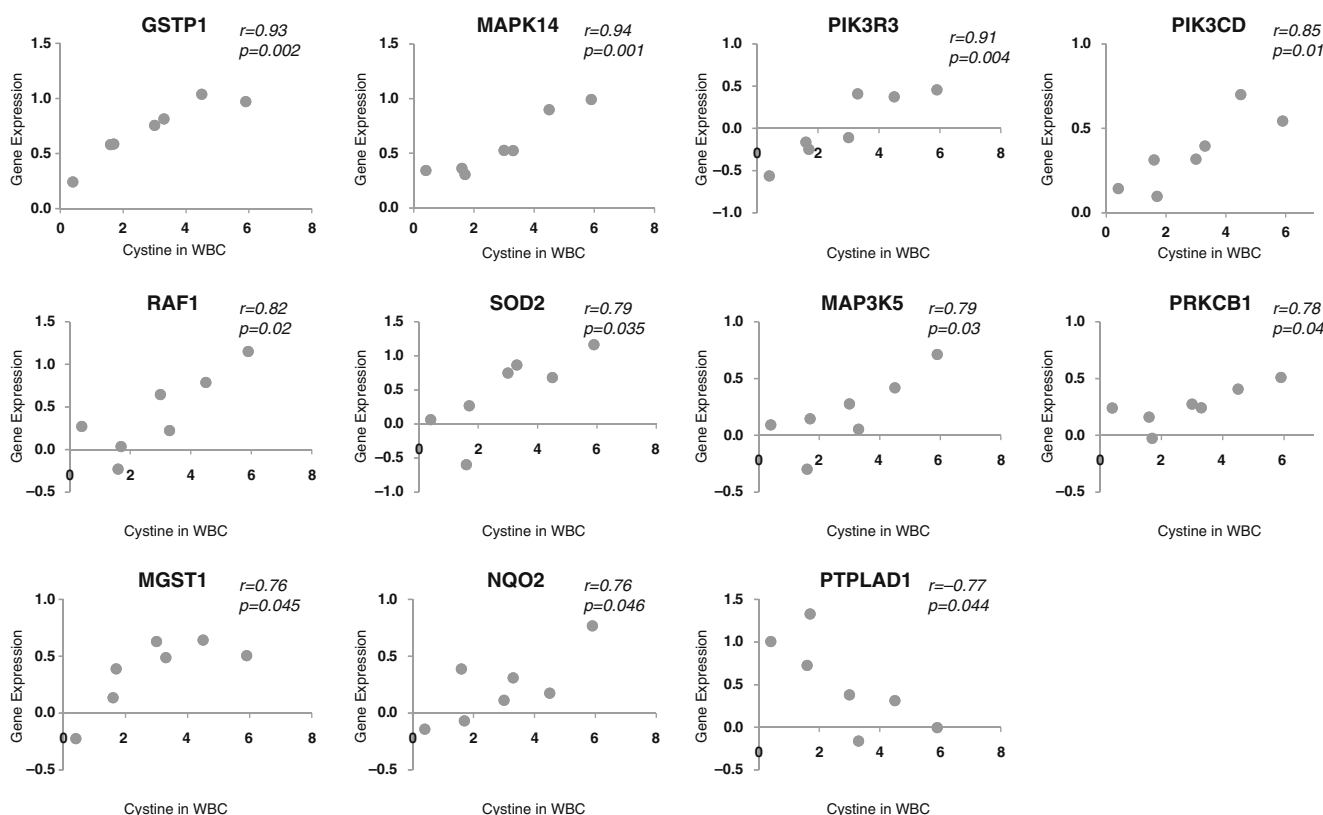


Fig. 2 Nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress response genes significantly associate with clinical white blood cell (WBC) cystine level. Eleven of 29 genes involved in NRF2-

mediated oxidative stress show a significant correlation between gene expression levels and WBC cystine levels ($p<0.05$, $r>0.76$)

pathways ($p=0.002$) were significantly downregulated in cystinosis. Canonical pathways that are significantly over-represented in this gene set are presented in Table 2 and ESM Fig. S2. Oxidative phosphorylation ($p=1.37\times 10^{-4}$) and apoptosis ($p=1.58\times 10^{-4}$) pathways were found to be highly significant by IPA and Pathway Express, respectively. B-cell receptor signaling ($p=6.89\times 10^{-7}$), antigen processing and presentation ($p=0.003$), ER stress ($p=5.18\times 10^{-3}$), mitochondrial dysfunction ($p=3.85\times 10^{-2}$), and nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress response ($p=2.95\times 10^{-6}$) pathways were also found to be highly significant by IPA. The signature of genes significantly associated with these pathways is highlighted in Table 2.

All clinical diagnoses of cystinosis were confirmed by measurement of WBC cystine levels, which were measured at the time of the blood collection for micro-

array analysis. Interestingly, 11 of 29 NRF2-mediated oxidative stress response genes significantly correlated with clinical WBC cystine levels in the blood samples from cystinosis patients ($p<0.05$, $r<1$; Fig. 2). WBC cystine levels compared with NRF2-mediated oxidative stress response gene expression demonstrated that gene expression levels were significantly increased or decreased as cystine levels increased or decreased. Detailed information on other relevant pathways and genes differentially expressed between cystinosis and control samples is given in ESM Figs. S3–S8.

Quantitative real-time PCR

Quantitative RT-PCR analysis was performed to validate the differentially expressed genes between cystinosis and control samples on microarray. Eleven of 35 differentially

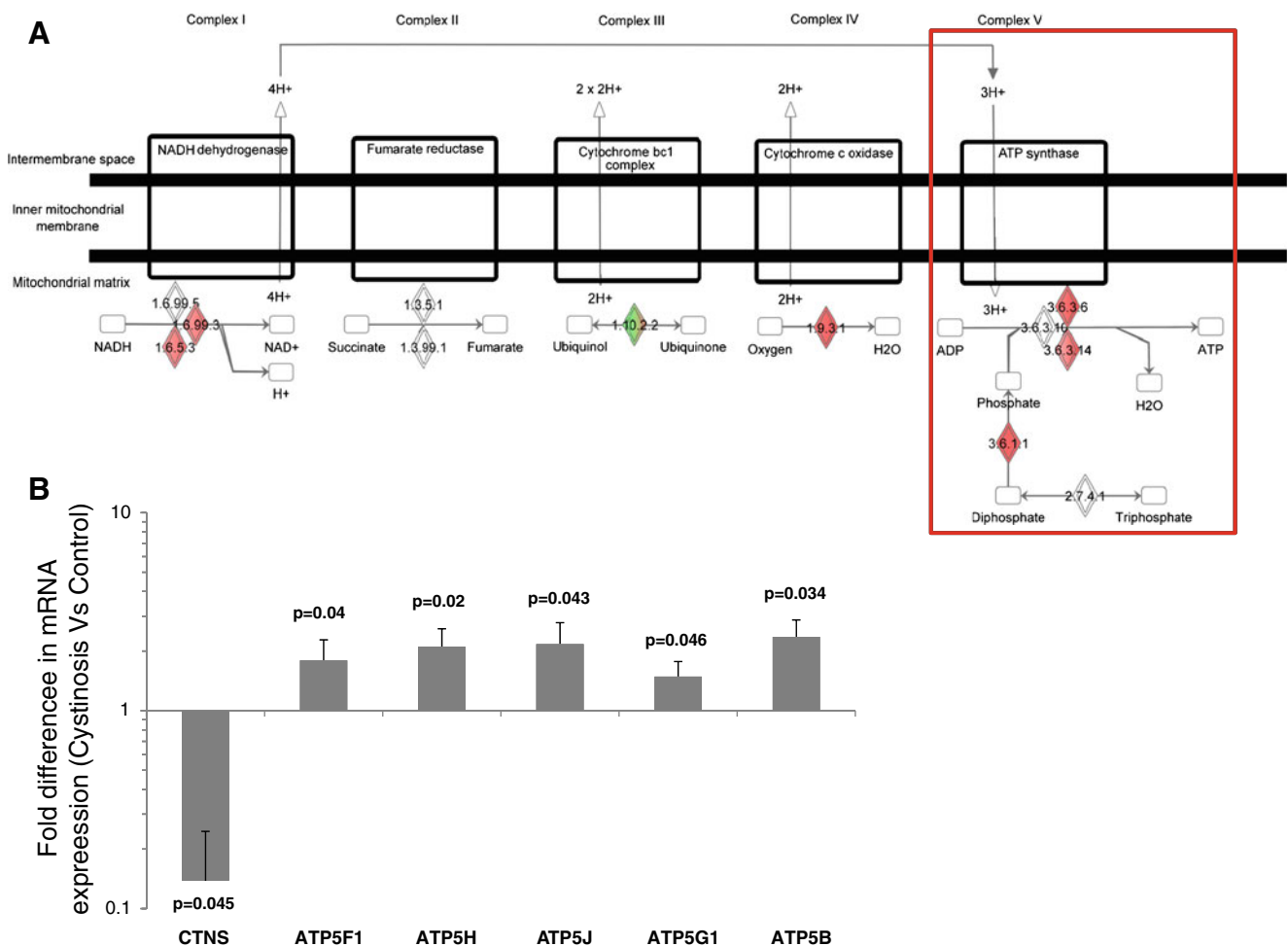


Fig. 3 Evaluation and validation of oxidative phosphorylation pathway in nephropathic cystinosis. **a** Ingenuity Pathway Analysis (IPA) identified oxidative phosphorylation ($p=1.37\times 10^{-4}$) as a significantly over-represented pathway in the differentially expressed gene set between cystinosis and control samples. **b** Quantitative real-time polymerase chain reaction (qRT-PCR) validation of microarray results

for complex V genes of mitochondrial oxidative phosphorylation pathway in the samples set ($n=7$ cystinosis and seven control samples). Cystinosis gene (*CTNS*) is used as a control gene, which is found to be significantly downregulated by qRT-PCR ($p=0.045$). *P* values were obtained by Student's *t* test

regulated genes identified from two highly significant pathways (apoptosis and oxidative phosphorylation) were selected for validation by qRT-PCR. The gene for cystinosis, *CTNS*, was included as a control in qRT-PCR. Quantitative RT-PCR confirmed the significant dysregulation of mitochondrial complex V genes and apoptosis genes in cystinosis (Fig. 3; Fig. 4). Significant downregulation of *CTNS*, noted on the microarray analysis, was again confirmed by qRT-PCR ($p=0.04$).

Electron and confocal microscopy

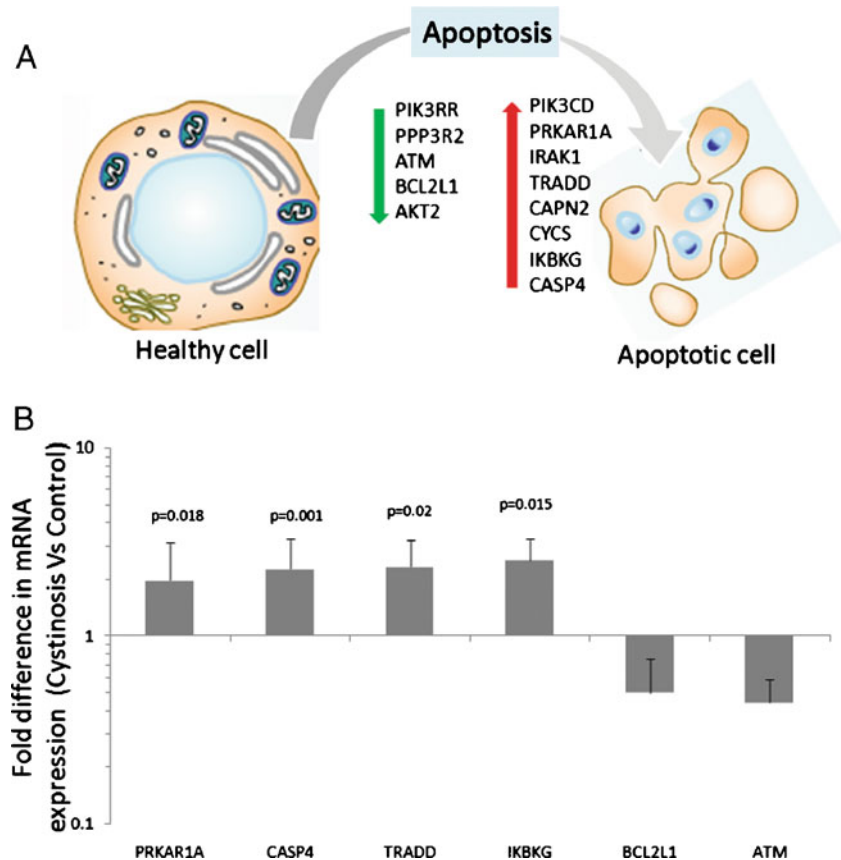
Ultrastructural analysis of cystinotic RPTE cells revealed anomalies in the mitochondria and ER in nephropathic cystinosis, confirming our IPA data that identified mitochondrial dysfunction ($p=3.85\times 10^{-2}$) and ER stress ($p=5.18\times 10^{-3}$) as significantly overrepresented pathways in the differentially expressed gene set between cystinosis and control samples (Fig. 5). In normal control RPTE cells, mitochondria were observed as structures with normal morphology (Fig. 5a), whereas in nephropathic cystinotic cells, mitochondria were present as abnormal structures with condensed and dark cristae (Fig. 5b). Intriguingly, nephropathic cystinotic RPTE cells presented ER with dilated ends, indicating ER stress in cystinosis (Fig. 5c, d). Additionally,

confocal microscopy of mitochondrial complex V protein (ATP-5F1) revealed the overexpression of complex V protein and abnormal fragmented morphology of mitochondria in nephropathic cystinotic RPTE cells (Fig. 5e, f).

Discussion

This is the first study to comprehensively characterize gene expression signature of peripheral blood in nephropathic cystinosis and sheds light on the critical pathways that may be dysregulated in key biological processes in this disease. There is significant enrichment of previously described and novel functional pathways in nephropathic cystinosis that involve cellular stress and targeted cell death. Increased apoptosis has been previously demonstrated (Park et al. 2002, 2006; Sansanwal et al. 2009) in cystinosis, and as expected, we could identify altered expression of various apoptotic genes in cystinosis (Table 2), including caspase-4 (*CASP4*), which has been recently shown to have higher expression in the cystinotic human kidney (Sansanwal et al. 2009); cytochrome-C (*CYCS*) (Acehan et al. 2002; Ott et al. 2002); TNFRSF1A-associated via death domain (*TRADD*) (Hsu et al. 1995); and calpain 2 (*CAPN2*, (m/II) large subunit).

Fig. 4 Validation of apoptosis pathway on cystinosis signature genes. **a** Apoptotic cell death and significant differentially expressed apoptosis genes in cystinosis, as identified by significance analysis of microarray (SAM). **b** Quantitative real-time polymerase chain reaction validation of microarray results for up- and downregulated apoptosis genes in nephropathic cystinosis. *P* values were obtained by Student's *t* test



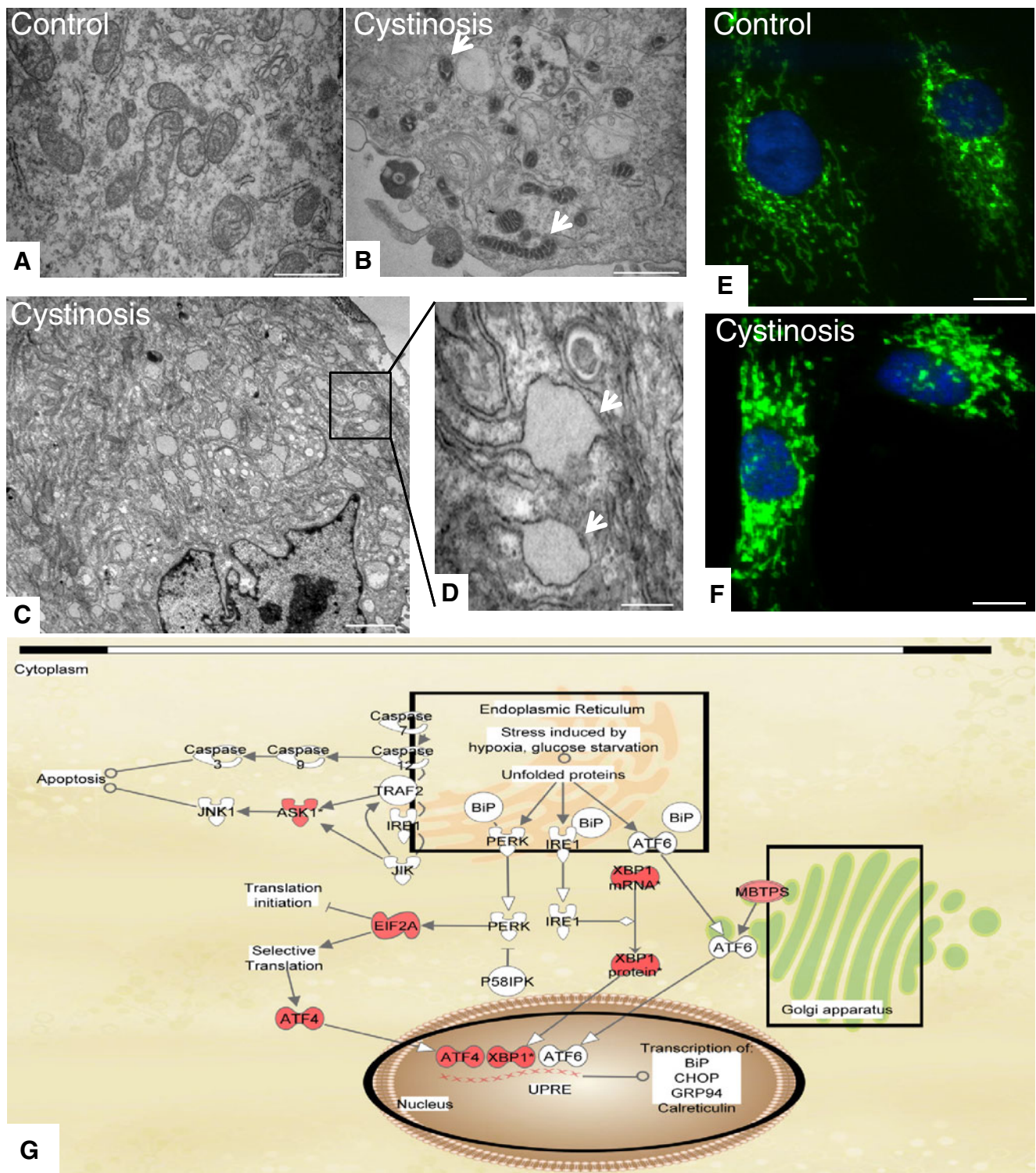


Fig. 5 Mitochondrial anomalies and endoplasmic reticulum (ER) stress evaluation in nephropathic cystinotic renal proximal tubular epithelial (RPTE) cells. **a–d** Electron microscopy of human normal RPTE cells (**a**) and nephropathic cystinotic RPTE cells (**b–d**). Dilated endoplasmic reticulum (ER) in cystinosis (**c**) is magnified and shown in *inset* (**d**). *White arrows* indicate abnormal mitochondria (**b**) and dilated ER (**d**) in cystinosis. *Bars*=1 μm in **a** and **b**; 2 μm in **c**, and 0.5 μm in **d**. *Magnifications* = $\times 20,500$ in **a** and **b**; $\times 6,600$ in **c**; $\times 40,000$ in **d**. **e, f** Confocal imaging of human normal RPTE cells (**e**)

and nephropathic cystinotic RPTE cells (**f**). Immunofluorescent staining specific for mitochondrial complex V subunit ATP-5F1 revealed abnormal morphology of mitochondria and higher expression of complex V protein in nephropathic cystinotic RPTE cells (**f**). *Magnifications*: $\times 63$. *Bars*=30 μm in **e** and **f**. **g** Ingenuity Pathway Analysis (IPA) identified ER stress ($p=5.18 \times 10^{-3}$) as a significantly overrepresented pathway in the differentially expressed gene set between cystinosis and control samples. Differentially expressed genes in cystinosis are shown in *red*

We recently showed (Sansanwal et al. 2010) that, in addition to apoptosis, autophagy—particularly mitophagy—is also a key regulator of tissue injury in cystinotic fibroblasts and renal tubular cells. In addition to the apoptosis genes noted above, lysosomal genes involved in autophagy also showed increased expression in cystinosis in this study. Some of the main lysosomal genes involved in autophagy are *LAMP2*, previously shown to be involved in the fusion of autophagosomes and lysosomes (Sansanwal et al. 2010; Stypmann et al. 2002), and *ABCA5*, which plays a role in processing autolysosomes (Kubo et al. 2005). *CALP2*, *ATG12*, and *ATF4* are also differentially expressed in cystinosis and are regulators of autophagosome formation (Demarchi and Schneider 2007; Demarchi et al. 2007; Namba et al. 2009; Whitney et al. 2009).

Cystinosis patients show differential changes in gene expression for certain immune-specific pathways relating to antigen presentation and processing. Several lysosomal storage disorders (LSD) have irregularities in the function of the immune system; a subset of LSD patients is predisposed to immune suppression, and some LSDs are predisposed to immune-system hyperactivity (Castaneda et al. 2008). There is insufficient evidence to determine the mechanism of altered immune response in cystinosis and its correlation with pathology and /or pathogenesis of the disease, but cystinosis patients are not particularly prone to more infections.

Oxidative phosphorylation is a major pathway affected in cystinosis and is responsible for the generation of reactive oxygen species (ROS). There is a highly coordinated gene set involved in oxidative phosphorylation in cystinosis (Table 2). It is interesting to observe these data, since production of ROS and induction of oxidative stress have previously been implicated in cystinosis (Rech et al. 2007). Notably, an elevated glutathione disulfide to total glutathione ratio was reported in cystinotic cells, suggesting increased oxidative stress in cystinotic cells (Levtchenko et al. 2005), and elevated oxidative stress has been associated with apoptosis (Corcoran et al. 1994). Aberrant energy production and electron transport-chain activity, ATP depletion, and altered redox status have been described in cells with cystine accumulation (Baum 1998; Levtchenko et al. 2006; Sansanwal et al. 2010; Wilmer et al. 2005). However, a previous report in cystinotic fibroblasts failed to detect the differences in mitochondrial complex V expression and activity (Wilmer et al. 2008). Interestingly, morphologically abnormal mitochondria in were reported as early as 1962 and 1971 in renal biopsies obtained from patients with nephropathic cystinosis (Jackson et al. 1962; Spear et al. 1971). Furthermore, aberrant mitochondrial morphology was also described in a murine cystinosis knock-out model and recently in RPTE cells from nephropathic cystinosis patients (Cherqui et al. 2002; Sansanwal et al. 2010).

In this study we identified for the first time that oxidative stress in cystinosis appears to be mediated by NRF2, the main inducible transcription factor involved in oxidative stress signaling (Abernathy et al. 1999; Achanzar et al. 2001; Cho et al. 2002; Kwak et al. 2001a,b); NRF2 helps to maintain intracellular redox homeostasis and limit oxidative damage in the cell (Cho et al. 2006; Kobayashi and Yamamoto 2006). The microarray analysis reveals a significant enrichment of groups of genes regulated by NRF2 in cystinosis (Table 2). In addition to its role in oxidative phosphorylation, NRF2 also regulates the expression of the cysteine–glutamate exchange transporter, which buffers cysteine influx against glutathione efflux (Sasaki et al. 2002). These findings are significant and very relevant in light of the fact that oxidative stress has been proposed to be a potential cause of proximal tubulopathy in cystinosis. NRF2-mediated oxidative stress response genes also significantly correlate with WBC cystine levels in cystinosis. Eleven of 29 genes involved in NRF2-mediated oxidative stress show a significant correlation between gene expression levels and WBC cystine levels ($p < 0.05$, $r > 0.76$), suggesting that this pathway can be further dysregulated when there is suboptimal cystine depletion, reflected by high WBC cystine levels.

Various organelle-specific genes are also dysregulated, highlighting a differential response to oxidative stress in the ER, mitochondria, and lysosomes in cystinosis. ER stress is a common manifestation in various lysosomal storage diseases (LSDs), including nephropathic cystinosis (Wei et al. 2008). Our electron microscopy results also confirm ER stress in cystinotic RPTE cells. Perhaps in response to this finding, many differentially expressed genes in cystinosis samples are associated with cell stress and ER signaling (Table 2) (Hwang et al. 2008; Vij et al. 2006; Wang et al. 2006). Also, NRF2 has been previously reported to participate in ER stress signaling (Weber et al. 2004; Xu et al. 2004).

Thus, this study provides novel insights into cellular stress and injury in patients with nephropathic cystinosis. Unique gene-expression signatures specific to NRF2-mediated oxidative stress response, mitochondrial dysfunction, cell death, ER stress, and immune dysregulation are identified in patients with nephropathic cystinosis. Further analysis of these genes and pathways may offer critical insights into the clinical spectrum of cystinotic patients and ultimately lead to novel links for targeted therapy.

Acknowledgements This work was supported by grants from the Cystinosis Foundation, Ireland, and the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA. We are indebted to Dr. L.C. Racusen (Department of Pathology, Johns Hopkins University) for generating an incredible resource of cystinotic renal proximal tubular epithelial cells; to Dr. Benedict Yen (Department of Pathology, University of

California, San Francisco) for support with electron microscopy experiments, and to Dr. William A. Gahl (Section on Human Biochemical Genetics, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health), for providing us with cystinotic RPTE cells and for helpful advice and review of the manuscript.

Funding Sources The work was supported by grants from the Cystinosis Foundation Ireland and the Intramural Research Program of the National Human Genome Research Institute, National Institute of Health.

References

- Abernathy CO, Liu YP, Longfellow D et al (1999) Arsenic: health effects, mechanisms of actions, and research issues. *Environ Health Perspect* 107:593–597
- Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell* 9:423–432
- Achanzar WE, Diwan BA, Liu J, Quader ST, Webber MM, Waalkes MP (2001) Cadmium-induced malignant transformation of human prostate epithelial cells. *Cancer Res* 61:455–458
- Anikster Y, Shotelersuk V, Gahl WA (1999) CTNS mutations in patients with cystinosis. *Hum Mutat* 14:454–458
- Baum M (1998) The Fanconi syndrome of cystinosis: insights into the pathophysiology. *Pediatr Nephrol* 12:492–497
- Castaneda JA, Lim MJ, Cooper JD, Pearce DA (2008) Immune system irregularities in lysosomal storage disorders. *Acta Neuropathol* 115:159–174
- Chen R, Li L, Butte AJ (2007) AILUN: reannotating gene expression data automatically. *Nat Methods* 4:879
- Cherqui S, Kalatzis V, Trugnan G, Antignac C (2001) The targeting of cystinosis to the lysosomal membrane requires a tyrosine-based signal and a novel sorting motif. *J Biol Chem* 276:13314–13321
- Cherqui S, Sevin C, Hamard G et al (2002) Intralysosomal cystine accumulation in mice lacking cystinosis, the protein defective in cystinosis. *Mol Cell Biol* 22:7622–7632
- Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, Kleeberger SR (2002) Role of NRF2 in protection against hyperoxic lung injury in mice. *Am J Respir Cell Mol Biol* 26:175–182
- Cho HY, Reddy SP, Kleeberger SR (2006) Nrf2 defends the lung from oxidative stress. *Antioxid Redox Signal* 8:76–87
- Corcoran GB, Fix L, Jones DP, Moslen MT, Nicotera P, Oberhammer FA et al (1994) Apoptosis: molecular control point in toxicity. *Toxicol Appl Pharmacol* 128:169–181
- Demarchi F, Schneider C (2007) The calpain system as a modulator of stress/damage response. *Cell Cycle* 6:136–138
- Demarchi F, Bertoli C, Copetti T, Eskelinen EL, Schneider C (2007) Calpain as a novel regulator of autophagosome formation. *Autophagy* 3:235–237
- Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95:14863–14868
- Gahl WA, Thoene JG, Schneider JA (2002) Cystinosis. *N Engl J Med* 347:111–121
- Hsu H, Xiong J, Goeddel DV (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 81:495–504
- Hwang SO, Boswell SA, Seo JS, Lee SW (2008) Novel oxidative stress-responsive gene ERS25 functions as a regulator of the heat-shock and cell death response. *J Biol Chem* 283:13063–13069
- Jackson JD, Smith FG, Litman NN, Yuile CL, Latta H (1962) The Fanconi syndrome with cystinosis. Electron microscopy of renal biopsy specimens from five patients. *Am J Med* 33:893–910
- Kalatzis V, Cherqui S, Antignac C, Gasnier B (2001) Cystinosis, the protein defective in cystinosis, is a H(+)-driven lysosomal cystine transporter. *EMBO J* 20:5940–5949
- Kalatzis V, Nevo N, Cherqui S, Gasnier B, Antignac C (2004) Molecular pathogenesis of cystinosis: effect of CTNS mutations on the transport activity and subcellular localization of cystinosis. *Hum Mol Genet* 13:1361–1371
- Khatri P, Desai V, Tarca AL, Sellamuthu S, Wildman DE, Romero R, Draghici S (2006) New onto-tools: promoter-express, nsSNPCounter and onto-translate. *Nucleic Acids Res* 34:W626–W631
- Kobayashi M, Yamamoto M (2006) Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. *Adv Enzyme Regul* 46:113–140
- Kubo Y, Sekiya S, Ohigashi M et al (2005) ABCA5 resides in lysosomes, and ABCA5 knockout mice develop lysosomal disease-like symptoms. *Mol Cell Biol* 25:4138–4149
- Kwak MK, Egner PA, Dolan PM et al (2001a) Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat Res* 480–481:305–315
- Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K et al (2001b) Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. *Mutat Res* 480–481:305–315
- Levtchenko E, de Graaf-Hess A, Wilmer M, van den Heuvel L, Monnens L, Blom H (2005) Altered status of glutathione and its metabolites in cystinotic cells. *Nephrol Dial Transplant* 20:1828–1832
- Levtchenko EN, Wilmer MJ, Janssen AJ, Koenderink JB, Visch HJ, Willems PH et al (2006) Decreased intracellular ATP content and intact mitochondrial energy generating capacity in human cystinotic fibroblasts. *Pediatr Res* 59:287–292
- Namba T, Homan T, Nishimura T, Mima S, Hoshiro T, Mizushima T (2009) Up-regulation of S100P expression by non-steroidal anti-inflammatory drugs and its role in anti-tumorigenic effects. *J Biol Chem* 284:4158–4167
- Nevo N, Chol M, Baillex A, Kalatzis V, Morisset L, Devuyst O et al (2010) Renal phenotype of the cystinosis mouse model is dependent upon genetic background. *Nephrol Dial Transplant* 25:1059–1066
- North American Pediatric Renal Trials and Collaborative Studies. NAPRTCS 2008 Annual Report. <https://web.emmes.com/study/ped/annlrept/annlrept.html>. Accessed May 2009.
- Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S (2002) Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci USA* 99:1259–1263
- Park M, Helip-Wooley A, Thoene J (2002) Lysosomal cystine storage augments apoptosis in cultured human fibroblasts and renal tubular epithelial cells. *J Am Soc Nephrol* 13:2878–2887
- Park MA, Pejovic V, Kerisit KG, Junius S, Thoene JG (2006) Increased apoptosis in cystinotic fibroblasts and renal proximal tubule epithelial cells results from cysteinylolation of protein kinase Cdelta. *J Am Soc Nephrol* 17:3167–3175
- Racusen LC, Wilson PD, Hartz PA, Fivush BA, Burrow CR (1995) Renal proximal tubular epithelium from patients with nephropathic cystinosis: immortalized cell lines as in vitro model systems. *Kidney Int* 48:536–543
- Rech VC, Feksa LR, Arevalo do Amaral MF et al (2007) Promotion of oxidative stress in kidney of rats loaded with cystine dimethyl ester. *Pediatr Nephrol* 22:1121–1128
- Sansanwal P, Kambham N, Sarwal MM (2009) Caspase-4 may play a role in loss of proximal tubules and renal injury in nephropathic cystinosis. *Pediatr Nephrol*
- Sansanwal P, Yen B, Gahl WA, Ma Y, Ying L, Wong LJ, Sarwal MM (2010) Mitochondrial autophagy promotes cellular injury in nephropathic cystinosis. *J Am Soc Nephrol* 21:272–283

- Sasaki H, Sato H, Kuriyama-Matsumura K et al (2002) Electrophile response element-mediated induction of the cystine/glutamate exchange transporter gene expression. *J Biol Chem* 277:44765–44771
- Spear GS, Slusser RJ, Tousimis AJ, Taylor CG, Schulman JD (1971) Cystinosis. An ultrastructural and electron-probe study of the kidney with unusual findings. *Arch Pathol* 91:206–221
- Stypmann J, Glaser K, Roth W et al (2002) Dilated cardiomyopathy in mice deficient for the lysosomal cysteine peptidase cathepsin L. *Proc Natl Acad Sci USA* 99:6234–6239
- Tibshirani R, Hastie T, Narasimhan B, Chu G (2002) Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci USA* 99:6567–6572
- Town M, Jean G, Cherqui S et al (1998) A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. *Nat Genet* 18:319–324
- Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98:5116–5121
- Vij N, Fang S, Zeitlin PL (2006) Selective inhibition of endoplasmic reticulum-associated degradation rescues DeltaF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. *J Biol Chem* 281:17369–17378
- Wang X, Venable J, LaPointe P et al (2006) Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. *Cell* 127:803–815
- Weber SM, Chambers KT, Bensch KG, Scarim AL, Corbett JA (2004) PPARgamma ligands induce ER stress in pancreatic beta-cells: ER stress activation results in attenuation of cytokine signaling. *Am J Physiol Endocrinol Metab* 287:E1171–E1177
- Wei H, Kim SJ, Zhang Z, Tsai PC, Wisniewski KE, Mukherjee AB (2008) ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders and are alleviated by chemical chaperones. *Hum Mol Genet* 17:469–477
- Whitney ML, Jefferson LS, Kimball SR (2009) ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. *Biochem Biophys Res Commun* 379:451–455
- Wilmer MJ, de Graaf-Hess A, Blom HJ et al (2005) Elevated oxidized glutathione in cystinotic proximal tubular epithelial cells. *Biochem Biophys Res Commun* 337:610–614
- Wilmer MJ, van den Heuvel LP, Rodenburg RJ et al (2008) Mitochondrial complex V expression and activity in cystinotic fibroblasts. *Pediatr Res* 64:495–497
- Xu W, Liu L, Charles IG, Moncada S (2004) Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response. *Nat Cell Biol* 6:1129–1134