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Report

Identification of Genes Affecting Cd Toxicity in HK-2 Cells

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Cadmium (Cd) is an environmental toxic heavy metal that predominantly causes renal failure. Although changes in gene expression are important factors affecting Cd toxicity, the genes that determine Cd toxicity have not been identified. In this study, we tested 36 genes that are highly expressed in the kidney for their effects on Cd toxicity. After human proximal tubular cells (HK-2 cells) were transfected with small interfering RNAs (siRNAs) targeting these genes, Cd toxicity was examined. The expression of the five genes selected from the primary screen was knocked down and the effect on Cd toxicity evaluated. The knockdown of *CRYAA* and *DPYS* significantly enhanced Cd toxicity, but not the toxicity of mercury compounds. The CRYAA protein plays a chaperone role and DPYS protein regulates nucleic acid metabolism. The regulation of *CRYAA* and *DPYS* expression may affect the Cd renal toxicity.

Key words cadmium, kidney, enriched gene, screening

INTRODUCTION

Environmental pollution caused by heavy metals is increasing around the world.[1,2] Importantly, heavy metal exposure has various adverse health effects in humans.[3] Cadmium (Cd) is the one of the toxic heavy metals contaminating the environment.[4] The primary effects of Cd poisoning are acute hepatic toxicity and chronic renal and bone toxicity.[4] Cd is ingested daily in humans by diverse foods, including rice, vegetables, and seafood, and by smoking.[4,5] With dietary exposure over a lifetime, Cd accumulates in the kidney and liver because the biological half-life of Cd is very long (15–30 years).[6] Notably, the renal dysfunction diagnosed in elderly people may be exacerbated by Cd accumulation. Cd causes cell death through necrosis, apoptosis, abnormal autophagy, the disruption of cell-to-cell adhesion, and the generation of reactive oxygen species.[7,8] In previous studies, we have demonstrated that the disruption of various genes regulated Cd toxicity in kidney-derived cultured cells. In particular, Cd has been shown to suppress the activities of the FOXF1, YY1, and ARNT transcription factors and to induce apoptosis by reducing the expression of their downstream genes.[9-11] These findings suggest that variations in gene expression in the kidney are important determinants of Cd toxicity. However, the basis for individual differences in the onset of Cd-induced nephrotoxicity remains unclear. Therefore, to evaluate the health effects of Cd exposure, the kidney-expressed genes that determine Cd sensitivity must be identified. The candidate genes were selected for analysis from The Human Protein Atlas (https://www.proteinatlas.org).

MATERIALS AND METHODS

Cell Culture and Treatment Human proximal tubular cells (HK-2 cells) were purchased from the American Type Culture Collection (Manassas, MA, USA), and cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12; Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified incubator under CO_2 (5%). The medium contains 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 25 µg/mL streptomycin (DS Pharm, Osaka, Japan), 25 U/mL penicillin (DS Pharm), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 1% insulin–transferrin–selenium X (Gibco), and 5 ng/mL hydrocortisone (Sigma-Aldrich).

Gene Screening Three small interfering RNAs (siRNAs) with different sequences were designed for each gene. The siRNAs were purchased from Ambion (Grand Island, NY, USA). The control siRNA (Silencer Select Negative Control No. 1 siRNA) was also purchased from Ambion. siRNA transfection was performed with Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen, Grand Island, NY, USA). A mixture of the three siRNAs directed against a specific gene [1 nM each siRNA sequence, 0.2% Lipofectamine™ RNAiMAX, and 10% Opti-MEM™ (Gibco), n = 3] was incubated in a 96-well plate for 15 min. HK-2 cells (250 cells/mm^2) were added into the siRNA mixture. After transfection for 48 h, the HK-2 cells were treated with $CdCl_2$ (Fujifilm Wako Pure Chemical Co., Tokyo, Japan) in serum-free culture medium. After treatment for 12 h, the Cd solution was replaced with FBS-supplemented medium containing alamarBlue® (10%; Invitrogen). The HK-2 cells were incubated for 4 h at 37°C. Fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 595 nm with a SpectraMax® iD3 micro-

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plate reader (Molecular Devices, San Jose, CA, USA).

RNA Extraction The cells were washed twice with ice-cold phosphate-buffered saline (–) [PBS(–)] (Gibco) and the total RNA was extracted with the PureLink™ RNA Mini Kit (Ambion), according to the instructions of the manufacturer. The concentration and purity of the RNA were determined with a BioSpec-nano spectrophotometer (Shimadzu, Kyoto, Japan).

Real-time Reverse Transcription (RT)–PCR The total RNA was reversed transcribed to cDNA (complementary DNA) with the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara Bio, Shiga, Japan). Real-time PCR was performed with SYBR® Premix Ex Taq™ II (Perfect Real Time) (Takara Bio) using the Thermal Cycler Dice Real Time System (Takara Bio). The thermal cycling conditions were: hot-start for 10 s at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Gene expression was normalized to *GAPDH* mRNA levels. The oligonucleotide sequences of the primers used were: sense, 5′-CGGGACAAGTTCGTCATCTT-3′, and antisense, 5′-GTTGTGCTTTCCGTGGATCT-3′, for the human *CRYAA* gene; sense, 5′-GACCTGGAGCTGTACGAAGC-3′, and antisense, 5′-TTCTTTGCTCCCTCTGCAAT-3′, for the human *DPYS* gene; sense, 5′-GCACCGTCAAGGCTGAGAAC-3′, and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′, for the human *GAPDH* gene.

Cell Viability The Silencer Select Predesigned siRNAs (Ambion) targeting *CRYAA* were s3540, s3541, and s3542; and those targeting *DPYS* were s4266, s4268, and s223437. HK-2 cells were transfected with the siRNA mixture for 48 h. After the culture medium was discarded, the cells were treated with $CdCl_2$ and mercury compounds in serum-free culture medium. Mercuric chloride ($HgCl_2$) was purchased from Fujifilm Wako Pure Chemical Co. Methylmercury chloride (CH_3HgCl) was purchased from GL Sciences Inc. (Tokyo, Japan). After treatment, 10% alamarBlue® was added and the cells were incubated for 4 h at 37°C. Fluorescence was measured at an excitation wavelength of 540 nm and an emission wavelength of 595 nm with a SpectraMax® iD3 microplate reader.

Statistical Analysis Statistical analyses were performed with one- or two-way ANOVA. When the F value was significant ($P < 0.05$), Bonferroni's multiple t test was performed for

Table 1. Genes Screened for the Moderation of Cd Toxicity.
Transcript levels of these genes were at least four-fold higher in the kidney than in any other tissues.

Gene	Gene description	Chromosome	Protein class
AGMAT	Agmatinase	1	Enzymes, Predicted intracellular proteins
AQP6	Aquaporin 6	12	Predicted membrane proteins, Transporters
ATP6V0D2	ATPase H+ transporting V0 subunit d2	8	Predicted intracellular proteins
ATP6V1G3	ATPase H+ transporting V1 subunit G3	1	Predicted intracellular proteins, Transporters
BBOX1	Gamma-butyrobetaine hydroxylase 1	11	Enzymes, Predicted intracellular proteins
BHMT2	Betaine--homocysteineS-methyltransferase 2	5	Cancer-related genes, Enzymes, Predicted intracellular proteins
BSND	Barttin CLCNK type accessory beta subunit	1	Disease related genes, Potential drug targets, Predicted membrane proteins, Transporters
CALB1	Calbindin 1	8	Predicted intracellular proteins
CLDN16	Claudin 16	3	Disease related genes, Potential drug targets, Predicted membrane proteins, Transporters
CRYAA	Crystallin alpha A	21	Disease related genes, Plasma proteins, Predicted intracellular proteins
CTXN3	Cortexin 3	5	Predicted membrane proteins
DPYS	Dihydropyrimidinase	21	Predicted intracellular proteins
EGF	Epidermal growth factor	4	Cancer-related genes
FMO1	Flavin containing monooxygenase 1	1	Enzymes, Predicted membrane proteins
FXYD4	FXYD domain containing ion transport regulator 4	10	Predicted membrane proteins
GGACT	Gamma-glutamylamine cyclotransferase	13	Enzymes, Predicted intracellular proteins
HMX2	H6 family homeobox 2	10	Predicted intracellular proteins, Transcription factors
KCNJ1	Potassium voltage-gated channel subfamily J member 1	11	Disease related genes, FDA approved drug targets, Predicted membrane proteins, Transporters
LHX1	LIM homeobox 1	17	Cancer-related genes, Predicted intracellular proteins, Transcription factors
MCCD1	Mitochondrial coiled-coil domain 1	6	Predicted secreted proteins
MIOX	Myo-inositol oxygenase	22	Enzymes, Predicted intracellular proteins
NAT8	N-acetyltransferase 8 (putative)	2	Enzymes, Predicted membrane proteins
NOX4	NADPH oxidase 4	11	Predicted membrane proteins, Transporters
NPHS2	NPHS2, podocin	1	Disease related genes, Potential drug targets, Predicted membrane proteins, Transporters
NPR3	Natriuretic peptide receptor 3	5	Predicted intracellular proteins, Predicted membrane proteins
OR2T10	Olfactory receptor family 2 subfamily T member 10	1	G-protein coupled receptors, Predicted membrane proteins
OTOGL	Otogelin like	12	Disease related genes, Predicted intracellular proteins, Predicted secreted proteins
RNF152	Ring finger protein 152	18	Enzymes, Predicted intracellular proteins, Predicted membrane proteins
SOST	Sclerostin	17	Disease related genes, Predicted secreted proteins
TINAG	Tubulointerstitial nephritis antigen	6	Enzymes, Predicted intracellular proteins
TMEM174	Transmembrane protein 174	5	Predicted membrane proteins
TMEM207	Transmembrane protein 207	3	Predicted membrane proteins
TMEM27	Transmembrane protein 27	X	Predicted membrane proteins, Transporters
TMEM52B	Transmembrane protein 52B	12	Predicted intracellular proteins, Predicted secreted proteins
TMEM72	Transmembrane protein 72	10	Predicted intracellular proteins, Predicted membrane proteins
UMOD	Uromodulin	16	Disease related genes, Plasma proteins

post hoc comparison ($P < 0.05$). All statistical analyses were performed with Statcel3 (OMS, Saitama, Japan).

RESULTS AND DISCUSSION

We selected genes from The Human Protein Atlas website to analyze the effects of their expression on the renal toxicity of Cd. The site provides information about tissue-specific proteomes. A transcriptomic analysis of the kidney indicated that 460 genes show elevated expression in the kidney relative to other tissue types (https://www.proteinatlas.org/human-proteome/tissue/kidney). The website specified 54 genes with enriched renal expression; the mRNA levels of these genes are at least four-fold higher in the kidney than in any other tissues. Because we focused on the factors regulating intracellular pathways in this study, we selected 36 candidate genes, excluding the solute carrier (SLC) family genes, for screening (Table 1). In the primary screen, the Cd sensitivity of HK-2 cells was examined after their transfection with a mixture of the three siRNAs specific for each of these 36 genes for 12 h. The primary screen showed that the siRNA treatment targeting *AQP6*, *OTOGL*, and *SOST* conferred relatively strong resistance to Cd (Supplementary Fig. 1), whereas the siRNA treatment targeting *CRYAA* and *DPYS* conferred relatively high sensitivity to Cd (Supplementary Fig. 1).

To confirm that these genes are associated with Cd toxicity, the knockdown efficiency and sensitivity of the assay were examined further. The transfection of *AQP6*- and *SOST*-directed siRNAs did not affect Cd toxicity (Supplementary Fig. 2), whereas *OTOGL*-directed siRNAs increased Cd toxicity (Supplementary Fig. 2). However, we could not confirm the knockdown efficiency of these genes with real-time RT–PCR (data not shown). The primary screen test was conducted one time (n=1); therefore, the confirmation examinations with reproducibility may exhibit different results.

The transfection of the *CRYAA*- and *DPYS*-directed siRNAs significantly reduced the corresponding mRNA levels (Figs. 1A, 1C), and Cd toxicity was enhanced by the knockdown of *CRYAA* and *DPYS* expression (Figs. 1B, 1D). The effects of *CRYAA* and *DPYS* knockdown on the toxicity of mercury compounds in HK-2 cells were then examined. The toxicity of methylmercury chloride and mercuric chloride on HK-2 cells was not altered markedly by the knockdown of *CRYAA* or *DPYS* (Fig. 2). These findings suggest that *CRYAA*

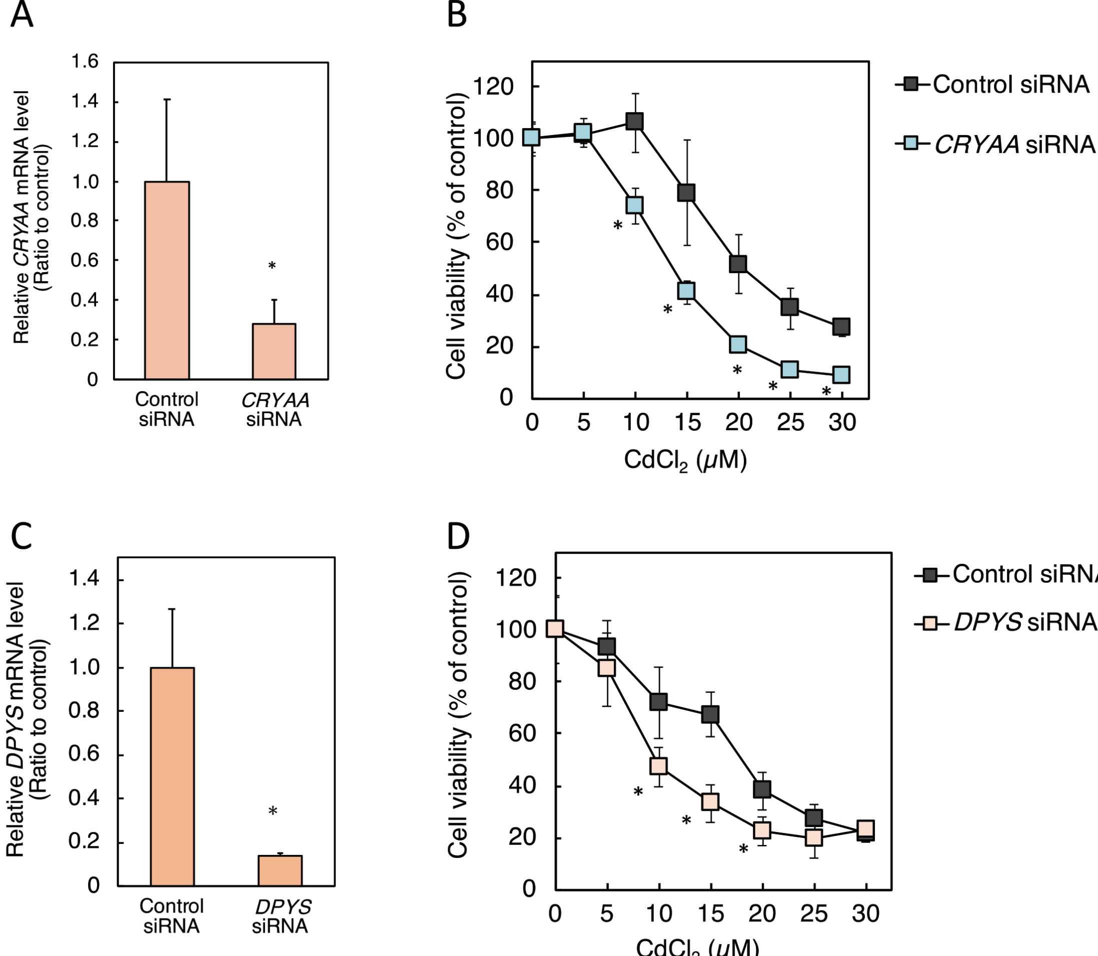

Fig. 1. Effects of *CRYAA* and *DPYS* Knockdown on the Viability of HK-2 Cells Treated with Cd

The efficiency of knockdown was examined after HK-2 cells were treated with siRNAs for 48 h. (**A, C**) mRNA levels were examined with real-time RT–PCR and normalized to *GAPDH* mRNA levels. Values are means ± SD (n = 3). (**B, D**) After treatment with siRNA for 48 h, HK-2 cells were treated with Cd for 12 h. Cell viability was examined with an alamarBlue® assay. Values are means ± SD (n = 5). *Significantly different from the corresponding control group, $P < 0.05$.

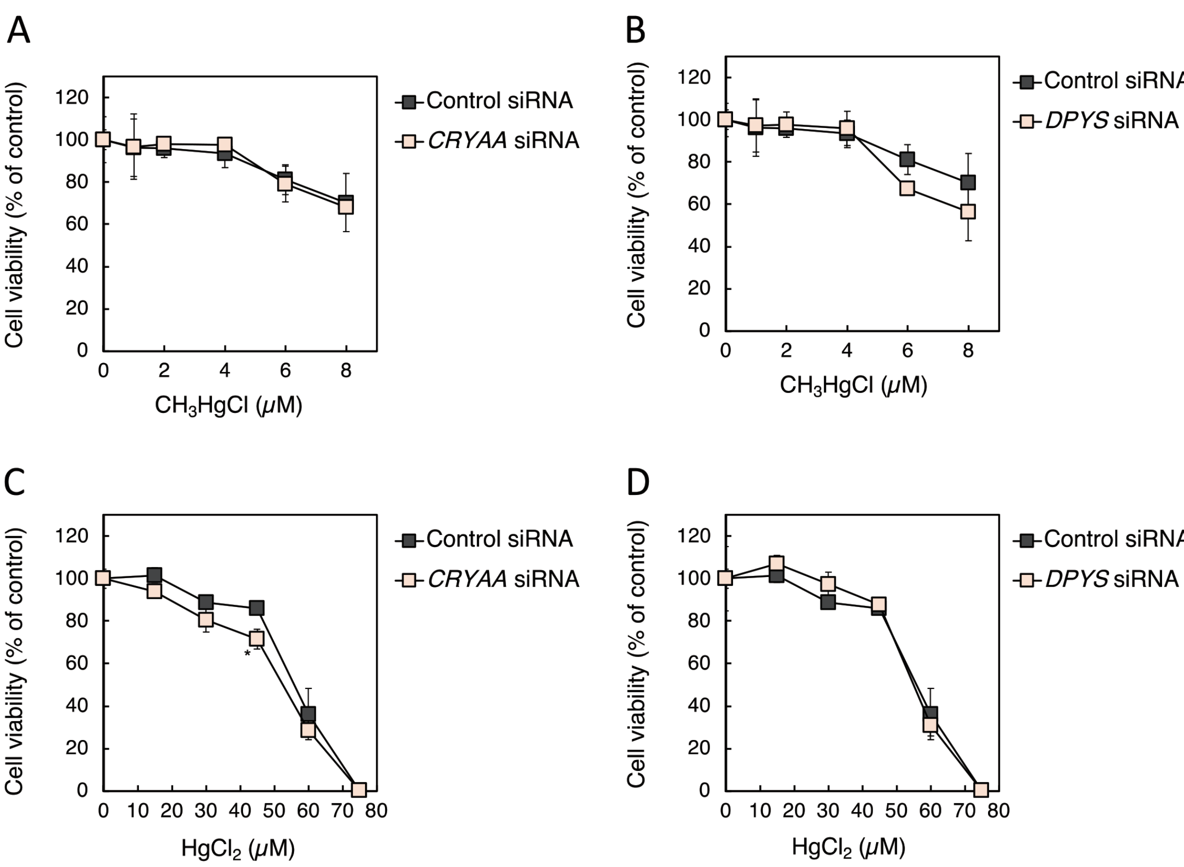

Fig. 2. Effects of *CRYAA* and *DPYS* Knockdown on the Viability of HK-2 Cells Treated with Mercury Compounds

(**A, B**) After treatment with siRNAs for 48 h, HK-2 cells were treated with methylmercury chloride (CH_3HgCl) for 12 h. (**C, D**) After treatment with siRNAs for 48 h, HK-2 cells were treated with mercuric chloride ($HgCl_2$) for 12 h. Cell viability was examined with an alamarBlue® assay. Values are means ± SD (n = 5). *Significantly different from the corresponding control group, $P < 0.05$.

and *DPYS* affect Cd toxicity through their expression changes in HK-2 cells.

CRYAA encodes an αA-crystallin protein that plays a role in the clarity and refractive properties of the lens.[12] αA-Crystallin also acts as a molecular chaperone.[13] In a previous study, we demonstrated that the aggregation of protein may affect Cd toxicity in HK-2 cells.[14] Therefore, CRYAA is considered to be involved in Cd toxicity through its chaperone function. *DPYS* encodes the protein dihydropyrimidinase, which is involved in the degradation of thymine and uracil and the regulation of nucleic acid metabolites.[15] Therefore, our findings suggest that the knockdown of *DPYS* enhances Cd toxicity, possibly *via* abnormalities in nucleic acid metabolites. This study is the first to report the involvement of the *CRYAA* and *DPYS* genes in Cd sensitivity in the kidney. Further research is required to clarify the novel pathways regulating the renal toxicity of Cd.

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Conflict of interest The authors declare no conflict of interest.

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