Characterization of Gene Expression Profiles of Metallothionein-Null Cadmium-Resistant Cells

Hitomi Fujishiro,[a] Satomi Okugaki,[a] Sachi Nagao,[a] Masahiko Satoh,[b] and Seiichiro Himeno*,[a]

[a]Laboratory of Molecular Nutrition and Toxicology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770–8514, Japan and [b]Department of Hygienics, Gifu Pharmaceutical University, Mitahorahigashi, Gifu 502–8585, Japan

(Received February 25, 2006; Accepted March 6, 2006; Published online March 10, 2006)

To understand metallothionein (MT)-independent mechanisms for cadmium (Cd) resistance in mammalian cells, we previously established MT-null Cd-resistant cells from embryonic fibroblast cells of MT-I and -II knockout mice. The Cd resistance of these cells was conferred primarily by a marked reduction of Cd accumulation. To identify genes responsible for Cd resistance as well as Cd transport, we carried out several DNA microarray analyses using cDNAs obtained from two clones of Cd-resistant cells and parental cells. A competitive hybridization of Cy3- and Cy5-probed cDNAs on a DNA chip was carried out with dye-swapping. After a careful examination of the reproducibility and reliability of the data obtained using five different chips, it was found that the expression of 78 genes was enhanced and that of 48 genes was reduced in Cd-resistant cells compared with those in parental cells. These genes include those involved in signal transduction, ubiquitin pathway, and cell-to-cell interactions. Several genes for transporters including solute carrier family transporters and ATP-binding cassette transporters were up- or down-regulated. The examination of mRNA levels using quantitative real-time PCR revealed that the expression of Slc39a14 encoding ZIP14, a member of the zinc transporter ZIP (ZRT-, IRT-like protein) family, was markedly down-regulated in both clones of Cd-resistant cells. Although it is not yet clear whether ZIP14 has the ability to transport Cd, these results suggest that the lowered expression of ZIP14 may be involved in Cd resistance in MT-null cells.

Key words —— cadmium, metallothionein, DNA microarray, gene expression, transport

INTRODUCTION

Metallothionein (MT) plays important roles in resistance to metal toxicity in mammalian cells. Especially, MT plays a critical role in the protection against cadmium (Cd) toxicity.[1)] When cells are exposed to Cd, MT synthesis is induced, and the increased MT serves as a scavenger for intracellular Cd ions. MT-null cells derived from MT-null mice are highly sensitive to Cd toxicity.[2,3)] Most Cd-resistant cell lines thus far established have shown enhanced production of MT.[4,5)] However, the presence of high concentrations of MT, which trap cellular Cd ions efficiently, has hindered precise understanding of Cd influx and efflux. In addition to the Cd transport system other Cd resistance factors irrelevant to MT have also been poorly elucidated. Thus the utilization of MT-null cells can give new insights into MT-independent Cd-resistance factors. For that purpose, we have established Cd-resistant cells from MT-null fibroblast cells derived from MT-I and -II knockout mice.[6)] The Cd resistance in these cells was conferred by a marked reduction of Cd accumulation. Both the uptake and efflux of Cd were changed in these Cd-resistant cells.[6)] Further analyses of the Cd-resistant MT-null cells indicated that the transport system for incorporation of both Cd and manganese (Mn) was not functioning in the Cd-resistant cells.[7)] Although it was reported that divalent metal transporter 1 (DMT1), the ferrous iron transporter, may participate in the transport of Cd and Mn, the properties of the Cd-resistant MT-null cells suggested the existence of a transport system different from DMT1. However, the entity of the Cd-uptake transporter that was suppressed in the Cd-resistant cells has not yet been clarified. Other non-

*To whom correspondence should be addressed: Laboratory of Molecular Nutrition and Toxicology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770–8514, Japan. Tel.: +81-88-622-9611 (ext. 5621); Fax: +81-88-655-3051; E-mail: himenos@ph.bunri-u.ac.jp

MT factors including Cd excretion and signal transduction may also be changed in these Cd-resistant cells and play roles in Cd resistance. Therefore, to identify the responsible genes for Cd resistance in MT-null cells, we compared the gene expression profiles between Cd-resistant and parental (P) cells using DNA microarray analysis.

MATERIALS AND METHODS

Cell Culture —— Cd-resistant MT-null cells (clones A7 and B5), and P cells were established previously.[6] A7, B5, and P cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin, and the media were changed every 2–3 days. It was confirmed that the Cd resistance of A7 and B5 cells was not lost even when cultured in Cd-free media.

Preparation of RNAs for DNA Microarray Analysis —— Total RNA was extracted and purified from cells using the SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.). DNA microarray analyses were performed using AceGene Mouse Oligo Chip 30K (Hitachi, Kanagawa, Japan), on which 30000 mouse genes were printed, according to the manufacturer's instructions. In brief, cDNA probes labeled with aminoallyl-dUTP (Ambion, Austin, TX, U.S.A.) were synthesized using $Oligo(dT)_{12–18}$ primer and SuperScript II (Invitrogen, Carlsbad, CA, U.S.A.), and purified using a QIA quick PCR purification kit (Quiagen K. K., Tokyo, Japan). The labeled test and reference cDNA probes were coupled with Cy3- and Cy5-monoreactive dye according to the manufacturer's instructions (Amersham Bioscience Inc., Tokyo, Japan) and were purified using microbio-spin columns (Bio-Rad Laboratories Inc., Tokyo, Japan). The arrays of DNA on the chips were hybridized with the labeled test and reference cDNA probes (cDNA synthesized from 100 μg of total RNA/chip) for 16 hr at 42°C. After being washed with 5 × standard saline citrate (SSC, NaCl 0.3 M, Na-citrate 0.3 M) and 0.1% sodium dodecyl sulfate for 5 min at 30°C, 0.5 × SSC for 5 min at 30°C, and 0.1 × SSC for 5min at 30°C, the chips were hybridized with Lucidea SlidePro (Amersham Biosciences) and scanned with CRBIO IIe (Hitachi Software Engineering, Japan). The digitized image data were processed with DNASIS Array software (Hitachi Software Engineering). The ratios of the intensity of Cy5 to that of Cy3 were calculated. Information on each gene on the chip was obtained from the NCBI database.

Determination of mRNA Levels in Real-time Reverse Transcription-PCR —— The same batch of RNA samples used for the DNA microarray was used for the real-time reverse-transcription (RT)-PCR analyses. The RT reaction was performed in a mixture containing Tris–HCl 50 mM, pH 8.3, KCl 70 mM, $MgCl_2$ 3 mM, dithiothreitol 10, 1 mM each of dNTP, 4 units of ribonuclease (RNase) inhibitor, 2 μg of total RNA, 0.5 μM $Oligo(dT)_{15}$ primer (Promega), and 5 units of reverse transcriptase in a total volume of 20 μl. The reaction was carried out at 37°C for 1.5 hr. The RT reaction mixtures were used directly for PCR amplification.[8] Quantitative real-time RT-PCR was performed using a TaqMan probe according to the procedure recommended by the manufacturer (Applied Biosystems, Foster City, CA, U.S.A.). For cDNA synthesis, 450 ng of total RNA was used. The forward and reverse primers and TaqMan probes for the ATP binding cassette transporter proteins and solute carrier family proteins were supplied by TaqMan Assay-on-Demand Products (Applied Biosystems). PCR amplifications were always performed using universal temperature cycles: 10 min at 94°C, followed by 35–45 two-temperature cycles (15 sec at 94°C and 1 min at 60°C). Fluorescence of PCR products was detected using an ABI Prism 7300/7500 Sequence Detector System (Applied Biosystems).

RESULTS

In the present study, we checked carefully the reliability and reproducibility of DNA microarray profiles by dye-swapping between Cy3 and Cy5 and by comparisons between two clones of Cd-resistant cells. The expression of Cy3- or Cy5-labeled cDNAs of A7 cells were competitively compared with Cy5- or Cy3-labeled cDNAs of P cells, respectively. Similar comparisons were made for B5 *versus* P cells with dye-swapping. The comparison between the Cy3-labeled cDNAs from P cells *versus* the Cy5-labeled cDNAs from B5 cells was performed twice because the results obtained in the first experiment showed relatively high background intensity, and the reproducibility of the data of this chip was much lower than that of other chips. From these five cross-labeled experiments, we selected the gene with high or low expression in Cd-resistant cells according to the following criteria. First, the genes with very low

intensity were omitted. Second, the genes showing the ratios (A7/P) of greater than 2.5 or less than 0.4 (1/2.5) on the chip with Cy3-labeled A7 cDNAs and Cy5-labeled P cDNAs were selected because this chip showed the highest reproducibility compared with other chips. Third, the genes that showed the ratios (A7/P, or B5/P) of greater than 2.5 or less than 0.4 on at least two chips were added. As a result, 126 genes with significant changes in expression were identified. The expression of 78 genes was enhanced and that of 48 genes was reduced in Cd-resistant cells compared with those of the P cells. Among these genes, genes with expression ratios of greater than 3.0 and less than 0.33 are listed in Table 1.

Although these include genes with unknown function, several genes involved in signal transduction, ubiquitin pathway, and cell-to-cell interactions, which have been known to participate in Cd resistance,[9] were enhanced or suppressed. Further studies on the roles of these genes may uncover novel mechanisms for Cd resistance irrelevant to MT.

Among transporter genes, only a few candidate genes were selected as enhanced or suppressed based on by the above-mentioned criteria (Table 1). However, if the basal expression levels of the genes for metal transporters are low, they could be overlooked in our DNA microarray analyses in which the reliability and reproducibility were examined stringently. Therefore all the data for the transporter genes from five chips were reanalyzed. The transporter genes are categorized into two superfamilies: the solute carrier family (Slc family) and ATP-binding cassette transporter. The expression profiles of all genes in these two families obtained by five analyses are shown in Fig. 1. We examined the reliability of the changes for all transporter genes again and selected Abcc9, Slc4a2, Slc7a5, Slc15a1, Slc35a1,

Table 1. List of the Genes with Expression Ratios (Cd-Resistant/Parental) of 3.0 or More (A), and 0.33 or Less (B) as Judged by the Criteria Described in the Text

A.

Accession No.	Gene Name	Definition
Ratio > 4.0		
NM_145940	D11Ertd498e	similar to hypothetical protein, unknown
NM_013754	Insl6	insulin-like 6
NM_033037	Cdo1	cysteine dioxygenase 1, cytosolic
AK003638	Akt1s1	AKT1 substrate1
AK010786	2410129E14Rik	homolog to tubulin beta chain
4.0 > Ratio > 3.0		
NM_025872	2310061A22Rik	riken cDNA, golgi transport 1 homolog B
NM_011104	Prkce	protein kinase c, epsilon
M57973	Cacna1c	cardiac l-type calcium channel
L36938	AI324046	Germline imuunoglobulin gamma constant region (IgG3)
NM_013688	Tcte1	t-complex-associated testis expressed 1
NM_012045	Pla2g2f	phospholipase A2, group IIF
Y18464	Ctsh	cathepsin h
AK006906	1700066M21Rik	riken cDNA, adult male testis cDNA, unknown
AF156490	Gabrg1	gamma-aminobutyric acid a receptor gamma 1 subunit
AK021238	Chm	Embryo whole body cDNA, unknown
X91617	Xrn1	5′-3′ exonuclease
AK015046	Kif2c	kinesin family member 2C
X06340	Cdh3	p-cadherin
NM_025711	Aspn	asporin
AF040953	Pirb	killer cell inhibitory receptor-like protein p91A
NM_015780	Cfhl1	complement factor H-related protein
BC024687	Vangl1	vang, van gogh-like 2
NM_010770	Matn3	matrilin 3
NM_016693	Map3k6	mitogen-activated protein kinase kinase kinase 6
AF006072	Polg2	mitochondrial dna polymerase accessory subunit

Table 1. Continued

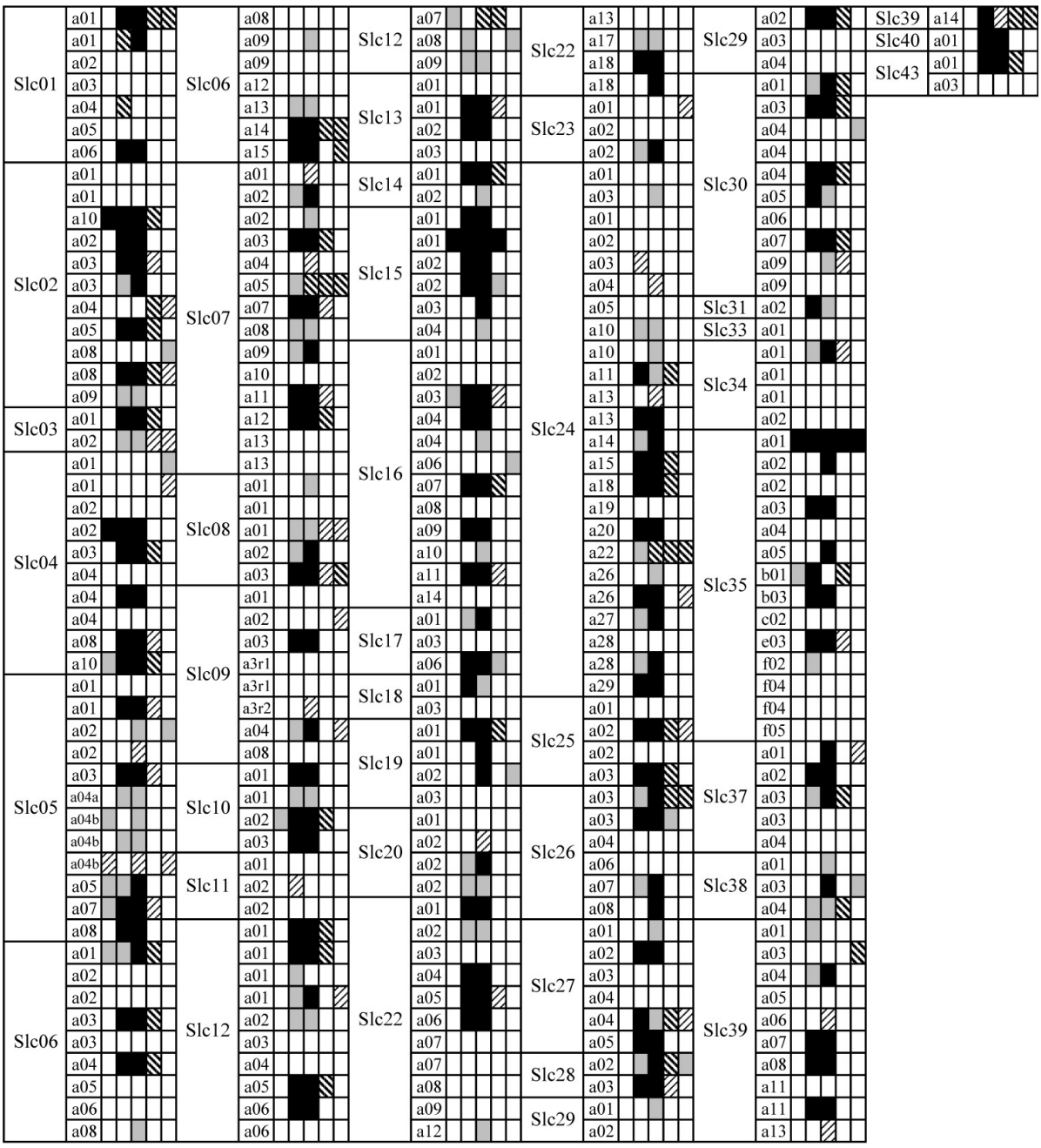
B.

Accession No.	Name	Definition
Ratio < 0.25		
NM_015783	G1p2	Interferon, alpha-inducible protein
NM_009311	Tac1	tachykinin 1
X52046	Col3a1	Collagen alpha 1
AK008830	2210404M20Rik	riken cDNA, hypothetical small nuclear ribonucleoprotein
NM_009926	Col11a2	procollagen, type XI, alpha 2 (col11a2)
NM_023422	Hist1h2bc	Histone1
AK008367	2010109K11Rik	riken cDNA, adult male small intenstine cDNA
X57984	Col9a1	pro alpha1 (IX) collagen chain
S54563	Col11a2	alpha 2(XI) collagen; col11a-2
AF331708	Npr2	natriuretic peptide receptor 2
AK015088	4930404N11Rik	hypothetical protein
BC023037	BC023037	similar to G protein pathway suppressor 2
$0.25 <$ Ratio < 0.33		
NM_008708	Nmt2	N-myristoyltransferase 2
AY073414	MOR239-2	olfactory receptor mor239-2
AK018601	9130008F23Rik	riken cDNA, adult male cecum cDNA, hypothetical protein, unknown
NM_007732	Col17a1	procollagen, type XVII, alpha 1
NM_009992	Cyp1a1	cytochrome p450, 1a1, aromatic compound inducible
NM_019709	Mbtps1	membrane-bound transcription factor protease, site 1
M18933	Col3a1	alpha-1 type-III collagen precursor
NM_023774	4930550L24Rik	riken cDNA, mage-k1 protein
BC005692	5430437P03Rik	riken cDNA, similar to hspc142 protein
AY073084	MOR255-1	olfactory receptor mor255-1
NM_021042	Abcc9	ATP-binding cassette, sub-family C(CFTR/MRP), member 9
Z23049	Ptprf	protein-tyrosine-phosphatase
AY073632	MOR279-1	olfactory receptor mor279-1
NM_130455	Grin3b	glutamate receptor, ionotropic, NMDA3B
NM_011404	Slc7a5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5; slc7a5
AY073715	MOR114-11	olfactory receptor mor114-11
NM_016968	Olig1	oligodendrocyte transcription factor 1
NM_013658	Sema4a	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4a
NM_007443	Ambp	alpha 1 microglobulin/bikunin

and Slc39a14 as up-regulated or down-regulated genes.

To examine the actual expression levels of these six genes, the mRNA levels of each gene relative to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in real-time PCR, and compared among A7, B5, and P cells. The changes in mRNA levels of these transporters showed a similar tendency to those obtained in the DNA microarray analyses, except for Slc7a5. Although Abcc9 is a transporter involved in efflux, the mRNA levels of Abcc9 were suppressed in Cd-resistant cells, suggesting no role of Abcc9 in Cd excretion. Slc4a2 encodes the sodium-independent chloride/bicarbonate anion exchanger (AE2), which is located widely in the plasma membrane and regulates intracellular pH and chloride, cell volume, and tonicity.[10] Slc7a5 encodes the large neutral amino acid transporter small subunit 2 (LAT 2) that transports neutral amino acid into the cells in the basolateral membrane of kidney proximal tubules and small intestine epithelia.[11] Slc15a1 encodes the H^+-coupled low-affinity pepetide transporter (PEPT1).[12] Slc35a1 encodes the cytidine 5′-monophosphate (CMP)-sialic acid trans-

a. SLC family



b. ABC transporter

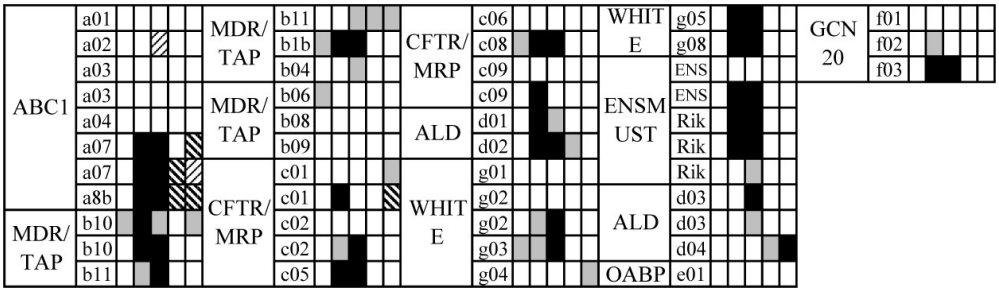


Fig. 1. Intensity Ratios (Cd-Resistant/Parental) of Genes Belong to Slc Family and ATP-binding Cassette (ABC) Transporters in Cd-Resistant Cells Examined in DNA Microarray Analyses

■, ≥ 2.0; ▩, 1.50–1.99; □, 0.67–1.49; ▨, 0.50–0.66; ▧, ≤ 0.49.

Table 2. Changes in mRNA Levels of Transporter Genes in Cd-Resistant Cells Revealed by Quantitative RT-PCR Analyses

Gene	Real time PCR	
	A7	B5
Abcc9	0.40 ± 0.07	0.41 ± 0.11
Slc4a2	1.68 ± 0.51	1.80 ± 1.49
Slc7a5	0.90 ± 0.24	2.47 ± 1.60
Slc15a1	6.67 ± 0.04	6.66 ± 0.34
Slc35a1	2.55 ± 0.06	2.06 ± 0.07
Slc39a14	0.08 ± 0.03	0.15 ± 0.14

The data were normalized by the levels of GAPDH, and expressed as the ratios to those of parental cells. Data were obtained from five experiments and presented as mean ± S.D.

porter located at the Golgi apparatus.[13] Although expression of these genes was enhanced, it remains to be clarified whether these transporters play any roles in Cd resistance.

As shown in Table 2, the most prominent change was observed in Slc39a14. The relative mRNA levels of Slc39a14 in the Cd-resistant/P cells were 0.08 and 0.15 in A7 and B5 cells, respectively. Slc39a14 encodes a gene for a zinc (Zn) transporter, ZIP14, a member of the ZIP (ZRT-, IRT-like protein) family. Although it is yet unknown whether ZIP14 can transport Cd in addition to Zn, the suppressed expression of ZIP14 in Cd-resistant cells that showed reduced uptake of Cd suggests that ZIP14 might be a candidate for the Cd transporter, or at least be involved in Cd resistance.

DISCUSSION

In a previous study, we established Cd-resistant cells from MT-null mouse cells and found that Cd accumulation in the resistant cells was markedly lower than in P cells.[6] Further analyses showed that the lowered Cd accumulation in the Cd-resistant cells was caused by the lower uptake and higher excretion of Cd than in P cells.[6] Kinetic studies on Cd uptake suggested the existence of a novel metal transporter with affinities for Cd, Mn, and Zn.[7] However, the metal transporter responsible for the suppressed uptake of Cd has not yet been identified. In the present study, to identify genes responsible for Cd resistance and transport, we carried out a series of DNA microarray analyses using cDNAs obtained from both Cd-resistant and P cells. Several candidate genes were found in the DNA microarray analyses with two clones of resistant cells. Quantitative real-time RT-PCR (Table 2) showed that Slc39a14, the gene for Zn transporter ZIP14, was markedly down-regulated in both clones of Cd-resistant cells. Although the actual contribution of ZIP14 to Cd transport should be examined in a future study, our results suggest that ZIP14 may be involved in Cd resistance in MT-null cells, possibly *via* the change in Cd transport.

ZIP transporters play major roles in cellular Zn uptake and intracellular Zn mobilization.[14] Recently, three reports have demonstrated that ZIP14 is expressed in mouse tissues and can transport Zn into cells.[15–17] Liuzzi *et al*. reported that ZIP14 mRNA was up-regulated by inflammation *via* the activation of interleukin-6 in the liver, suggesting a role of ZIP14 in inflammatory Zn deficiency.[18] However, no information has yet been available on whether ZIP14 participates in the transport of other metals such as Cd or Mn. The other transporters such as DMT1 (Slc11a2)[19] and ZIP8 (Slc39a8)[20] have been reported to have the ability to transport Cd, but the DNA microarray analysis in the present study did not show any significant changes in the expression of DMT1 or ZIP8 in MT-null Cd-resistant cells. In a previous study, we demonstrated that not only Mn but also Zn competed with Cd for incorporation into P cells *via* the high-affinity transport system for Cd and Mn.[7] Further studies are now on going to clarify whether other Zn transporters are involved in Cd transport.

In the present study, we conducted the DNA microarray analyses five times to identify changes in the gene expression in Cd-resistant cells strictly and carefully. It was demonstrated that the reproducibility of the microarray data was not high after dye-swapping. One of the reasons for this might be the difference in labeling efficiency between Cy3 and Cy5, especially when the intensity of the signal is low. As shown in Fig. 1, in some cases opposite results (high in one chip and low in another chip) were obtained for the same gene among the five analyses. However, the comparison of two resistant cell clones, dye-swapping, and a careful examination of the reproducibility and intensity of the signals have enabled us to identify several genes showing significant changes in expression. Among the transporter genes, we selected six candidates from DNA microarrays, and confirmed the actual changes in mRNA levels for five of them using quantitative

real-time PCR (Table 2). Nonetheless, the possibility could not be excluded that more important changes in the gene expression in Cd-resistant cells were overlooked in these analyses.

In conclusion, using a series of DNA microarray analyses and subsequent real-time PCR analyses to assess the molecular concomitants of Cd resistance using two clones of Cd-resistant MT-null cells, we found a marked down-regulation of the Slc39a14 gene in both cell lines. Although the role of Slc39a14 (ZIP14) in Cd transport is yet to be clarified, the present study suggested that the Zn transport system including ZIP14 may be involved in Cd transport or resistance.

Acknowledgements This work was partly supported by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

1) Suzuki, K. T. and Imura, N. and Kimura, M. (eds.) (1993) *Metallothionein III, Biological Roles and Medical Implications*, Birkhauser Verlag, Basel.
2) Lazo, J. S., Kondo, Y., Dellapiazza, D., Michalska, A. E., Choo, K. H. A. and Pitt, B. R. (1995) Enhanced Sensitivity to Oxidative Stress in Cultured Embryonic Cells from Transgenic Mice Deficient in Metallothionein I and II Genes. *J. Biol. Chem.*, **270**, 5506–5510.
3) Kondo, Y., Yanagiya, T., Himeno, S., Yamabe, D., Schwartz, D., Akimoto, M., Lazo, J. S. and Imura, N. (1999) Simian virus 40-transformed metallothionein null cells showed increased sensitivity to cadmium but not to zinc, copper, mercury or nickel. *Life Sci.*, **64**, PL145–PL150.
4) Grady, D. L., Moyzis, R. K. and Hildebrand, C. E. (1987) Molecular and cellular mechanisms of cadmium resistance in cultured cells. *Experientia*, **52**(Suppl.), 447–456.
5) Li, W., Kagan, H. M. and Chou, I. N. (1994) Alterations in Cytoskeletal Organization and Homeostasis of Cellular Thiols in Cadmium-Resistant Cells. *Toxicol. Appl. Pharmacol.*, **126**, 114–123.
6) Yanagiya, T., Imura, N., Kondo, Y. and Himeno, S. (1999) Reduced Uptake and Enhanced Release of Cadmium in Cadmium-Resistant Metallothionein Null Fibroblasts. *Life Sci.*, **65**, 177–182.
7) Yanagiya, T., Imura, N. S., Kondo, Y. and Himeno, S. (2000) Suppression of a high-affinity transport system for manganese in cadmium-resistant metallothionein null cells. *J. Pharmacol. Exp. Ther.*, **292**, 1080–1086.
8) Donai, H., Murakami, T., Amano, T., Sogawa, Y. and Yamauchi, T. (2000) Introduction and alternative splicing of isoform of Ca^{2+}/calmodulin-dependent protein kinase II during neural differentiation of P19 embryonal carcinoma cells and during brain development. *Brain Res. Mol. Brain Res.*, **85**, 189–199.
9) Othumpangat, S., Kashon, M. and Joseph, P. (2005) Eukaryotic translation initiation factor 4E is a cellular target for toxicity and death due to exposure to cadmium chloride. *J. Biol. Chem.*, **280**, 25162–25169.
10) Lecanda, J., Urtasun, R. and Medina, J. F. (2000) Molecular cloning and genomic organization of the mouse AE2 anion exchanger gene. *Biochem. Biophys. Res. Commun.*, **276**, 117–124.
11) Rossier, G., Meier, C., Bauch, C., Summa, V., Sordat, B., Verrey, F. and Kuhn, L. C. (1999) LAT2, a new basolateral 4F2hc/CD98-assoiated amino acid transporter of kidney and intestine. *J. Biol. Chem.*, **274**, 34948–34954.
12) Fei, Y. J., Sugawara, M., Liu, J. C., Li, H. W., Ganapathy, V., Ganapathy, M. E. and Leibach, F. H. (2000) cDNA structure, genomic organization, and promoter analysis of the mouse intestine peptide transporter PEPT1. *Biochem. Biophys. Acta*, **1492**, 145–154.
13) Eckhardt, M., Muhlenhoff, M., Bethe, A. and Gerardy-Schahn, R. (1996) Expression cloning of Golgi CMP-sialic acid transporter. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 7572–7576.
14) Taylar, K. M., Morgan, H. E., Johnson, A. and Nicholson, R. I. (2004) Structure function analysis of HEK4, a member of the new LIV-1 subfamily of zinc transporters. *Biochem. J.*, **377**, 133–139.
15) Gaither, L. A. and Eide, D. J. (2000) Functional expression of the human hZIP2 zinc transporter. *J. Biol. Chem.*, **275**, 5560–5564.
16) Gaither, L. A. and Eide, D. J. (2001) The human ZIP1 transporter mediates zinc uptake in human K562 erythroleukemia cells. *J. Biol. Chem.*, **276**, 22258–22264.
17) Dufner-Beattie, J., Langmade, S. J., Wang, F., Eide, D. and Andrews, G. K. (2003) Structure, function, and regulation of a subfamily of mouse zinc transporter genes. *J. Biol. Chem.*, **278**, 50142–50150.
18) Liuzzi, J. P., Louis, A. L., Raymond, S. R., Banchard, K., Aydemir, T. B., Knutson, M. D., Ganz, T. and Cousins, R. J. (2005) Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 6843–6848.

19) Dalton, T. P., He, L., Wang, B., Miller, M. L., Jin, L., Stringer, K. F., Chang, X., Baxter, C. S. and Nebert, D. W. (2005) Identification of mouse SLC39A8 as the transporter responsible for cadmium-induced toxicity in the testis. *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 3401–3406.
20) Gunshin, H. (1997) Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* (London), **388**, 482–488.