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Identification of Differentially Expressed Genes by Exposure of Methylmercury in Neuroblastoma Cell Line Using Suppression Subtractive Hybridization (SSH)

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Abstract

Methylmercury (MeHg), one of the heavy metal compounds, can cause severe damage to the central nervous system in humans. Many reports have shown that MeHg is poisonous to human body through contaminated foods and has released into the environment. Despite many studies on the pathogenesis of MeHg-induced central neuropathy, no useful mechanism of toxicity has been established so far. This study, using of suppression subtractive hybridization (SSH) method, was performed to identify differentially expressed genes by MeHg in SH-SY5Y human neuroblastoma cell line. We prepared to total RNA from SH-SY5Y cells treated with solvent (DMSO) and 6.25 μM (IC_{50}) MeHg and performed forward and reverse SSH. Differentially expressed cDNA clones were screened by dot blot, sequenced and confirmed that individual clones indeed represent differentially expressed genes with real time RT-PCR. These sequences were identified by BLAST homology search to known genes or expressed sequence tags (ESTs). Analysis of these sequences may provide an insight into the biological effects of MeHg in the pathogenesis of neurodegenerative disease and a possibility to develop more efficient and exact monitoring system of heavy metals as ubiquitous environmental pollutants.

Keywords: Methylmercury, human neuroblastoma cell line, suppression subtractive hybridization (SSH), Differentially expressed genes

Mercury can be converted in the environment by microorganisms and natural chemical process into the organic form, methylmercury (MeHg), which is especially toxic. MeHg accumulates in fish, and ultimately in humans through the food chain cycle. MeHg levels for most fish range from less than 0.01 ppm to 0.5 ppm. The U.S. Environmental Protection Agency (USEPA) established a reference dose for MeHg of 0.6 μg/kg per day[1]. MeHg is efficiently absorbed into the body and crosses both the blood-brain barrier and the placental barrier. Its biological half-life in humans is about 70 days. It has also been shown that mercury is selectively concentrated in the human brain. Also, the immature central nervous system (CNS) is extremely sensitive to MeHg neurotoxicity and the fetal brain may be affected even if the mother shows no signs of poisoning. The increasing daily fish consumption as a part of a healthy diet may result in chronic low-level dietary intake of MeHg and thus poses a significant toxicological problem, especially to susceptible individuals, such as developing embryos and fetuses.

High dose exposure of MeHg may result in cerebral palsy, blindness, deafness, and severe mental retardation, due to degeneration and decrease in number of its nerve cells[2], disappearance of granule cells and reduction of Purkinje cells and white matter astrocytosis[2]. And *in vitro* assay, relatively high (5-10 μM) MeHg levels cause the rapid impairment of mitochondrial activity, de-energization of mitochondria and plasma membrane lysis, resulting in extensive necrotic death with early onset[3].

A number of molecular targets and mechanisms have been proposed to be implicated in its neurotoxic effects. The high affinity of MeHg for thiol groups makes proteins and peptides bearing cysteines susceptible to structural and functional modification by MeHg in all subcellular compartments[4,5]. In recent, our laboratory elicited that the target protein may be selenoprotein W regulated transcription levels dependent of glutathione depletion in human neuronal cells exposed to low level of MeHg[6]. Also, we identified that MeHg affects the transcription of a number of genes depend on exposed time (in published). However, these changes of gene expression by low level-MeHg treatment were not drastically differential. So, in this study, we attempted suppression subtractive hybridization (SSH) method to identify differentially expressed genes (DEGs) by MeHg in human neuronal

cells treated to high level-MeHg for 6 hr.

There are several PCR-based approaches to analyze changes in gene expression. Several different techniques for the isolation of DEGs have been developed, including differential display[7,8], representational difference analysis[9], and subtractive hybridization[10,11]. A major drawback of these methods is that they perform poorly for rare transcripts[11]. Data from microarray hybridizations are also unreliable for genes expressed at low levels[12]. SSH allows for the generation of subtracted cDNA populations and overcomes the problem of differences in mRNA abundances by incorporating a hybridization step that normalizes sequence abundance during the subtraction[13,14]. It dramatically increases the probability of obtaining low-abundance DEGs and simplifies the analysis of the subtracted cDNA libraries.

Thus our experimental approach allowed us to identify DEGs related to high level-MeHg treatment in human neuronal cells, without bias toward abundantly expressed messages. This study could elucidate the mechanism of the neurotoxicity by MeHg and lead to new aspect of monitoring of MeHg through using these DEGs as biomarker.

Results

Cytotoxicity of MeHg in SH-SY5Y Human Neuronal Cells

Dose dependent cell viability curves were obtained after from 3 hr to 7 days of exposure to MeHg in SH-SY5Y cells, a human neuroblastoma cell line (Fig. 1A). At 6 hr, MeHg exhibited a median inhibition concentration (IC_{50}) of 6.25 μM. After 1 μM and 6.25 μM MeHg treatment for 6 hr, morphology of SH-SY5Y cells was observed (Fig. 1B). The proliferation of SH-SY5Y cells was inhibited by MeHg in a dose-dependent manner (Fig. 1A), and the morphology of the treated cells was not changed until 1 μM treatment (Fig. 1B).

Identification of the Genes Induced by MeHg in Human Neuroblastoma SH-SY5Y Cells in SSH

We used the concentration of 6.25 μM MeHg for SSH to identify the MeHg-inducible genes related to the neurotoxicity. Following SSH, 96 clones were chosen from the forward subtraction (MeHg treatment subtracted control) and 127 clones were chosen from the reverse subtraction (control subtracted MeHg treatment). And these clones were randomly chosen and rescreened for differential gene expression by dot-blot hybridization, and were then sequenced. An example of nitrocellulose membrane probed for dot-blot screening is shown in Fig. 2. The screening indicated that 24 clones were up-regulated and 11 clones were down-regulated, as shown in Table 2 and 3; the clones were then sequenced. Of these, clones have repeated or similar sequences were eliminated.

Table 1. Primer sequences used for real time RT-PCR of differentially expressed genes by MeHg.

Gene	Primer sequences
Hsp90-F	5′-CATGATGTTAACTTTGTGTGGTCTA AAG-3′
-R	5′-TCAAGATACAGCTCAGAACACTTC AA-3′
BPA-1-F	5′-TGACAGATGCTTGGCCAGAA-3′
-R	5′-AGGACAATAACTACCATGGCTCTCA -3′
AL133511-F	5′-AAGAAATGGATAAATTCCTAGACAC ACA-3′
-R	5′-TTGATCTCATTACTTGTTATTGGTCT GTT-3′
CNAP1-F	5′-CCCAGGCTTTGATTCAGAATCT-3′
-R	5′-ACAAACTCACAGAGAATTTCCTCAA G-3′
LAPTM4A-F	5′-GTTTGGAACTGCTATAAATACATCA ACAA-3′
-R	5′-AACGTACTGAGGAGGTGCTTCAA-3′
LEC1-F	5′-TCAATCCAACTGTCCGCAAA-3′
-R	5′-AGGCTCCGGTAAATGATGAACA-3′
PDK-F	5′-TCTCCCGAACTAGAACTTGAAGAAC T-3′
-R	5′-CATGTGATAGAGATGGGATGGTACA T-3′

Hsp90: 90-kDa heat-shock protein, BPA-1: mRNA for brain peptide A1, AL133511: Human DNA sequence from clone RP1-4K15, CNAP1: chromosome condensation-related SMC-associated protein 1, LAPTM4A: lysosomal-associated protein transmembrane 4, LEC1: lectomedin-1 gamma, PDK1: pyruvate dehydrogenase kinase, isoenzyme 1, F: forward primer sequence, R: reverse primer sequence. All primer sequences were determined with established GenBank sequences.

Real-Time RT-PCR Analysis of Identified Genes in MeHg-Treated SH-SY5Y Cells

We next performed quantitative real-time RT-PCR analysis to further confirm the differential expression of the identified genes. Six function-known genes and 1 human EST among 35 clones were examined. As shown in Fig. 3, MeHg treatment induced 90-kDa heat shock protein (Hsp90) mRNA expression in a dose-dependent manner. The expression level of Hsp90 mRNA in the treated cells was approximately 3.3-fold higher than that in the untreated cells, 6 hr

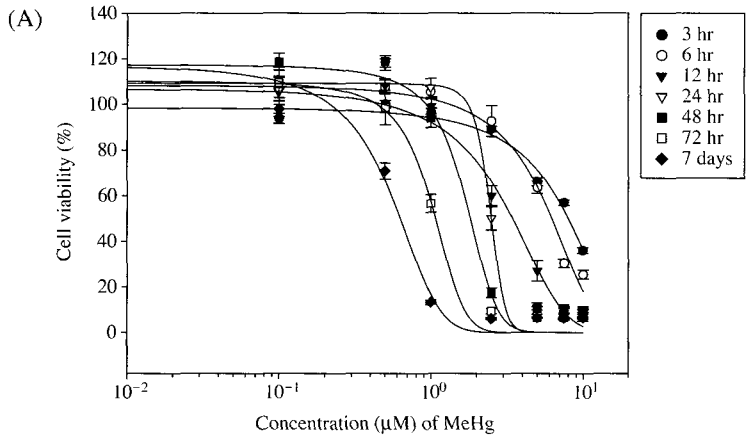

Fig. 1. Effect of Methylmercury on SH-SY5Y Cells Viability. (A) Dose- and time-response curve assessed at each time points by the MTT assay after treatment. SH-SY5Y cells grown for 2 days in 96-well plates were exposed to MeHg in culture medium. IC_{50} value of MeHg was calculated to 6.25 μM at 6 hr treatment. Values are expressed as percentage of corresponding controls (mean ± SD). (B) Morphology of MeHg treated SH-SY5Y cells.

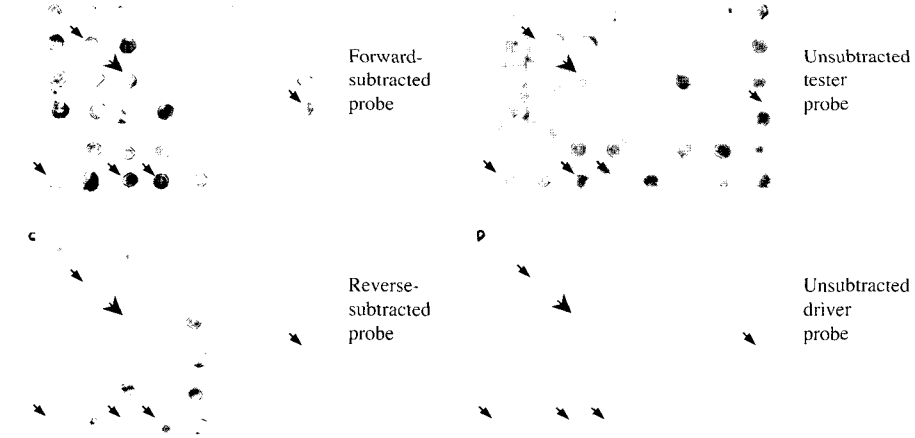

Fig. 2. Differential screening of cDNA library using dot blots. The cDNA clones randomly picked from forward subtraction of SSH were cultured in four identical nitrocellulose membranes and then separately hybridized to tester, driver, unsubtracted tester and unsubtracted driver probe. The arrow (thick and thin) shows the positive clones with different levels of expression between two conditions. The thick arrow represents 90 kDa heat shock protein.

Forward	Reverse	Unsubtr. tester	Unsubtr. driver	Interpretation
+	−	+	−	Almost always correspond to DEG
+	−	−	−	Strong candidates for DEG
+	−	+	+	Difficult to interpret : artifact
++	+	+	+ or −	>5 fold probably correspond DEG
+	+	+	+	Never DEG
−		−	−	Non-DEG

Table 2. Summary of up-regulated genes by MeHg-treatment in human neuroblastoma cells showing accession number and gene name of SSH cDNA clone.

Clone #	Access #	Up-regulation suspected gene name
1	BC018704	Homo sapiens phosphotidylinositol transfer protein, beta
2	AC090692	Homo sapiens chromosome 11, clone RP11-698N11
3	AB037773	Homo sapiens mRNA for KIAA1352 protein
4	U24166	Homo sapiens EB1 mRNA
10	BC002387	nucleosome assambly protein
15	AC025594	Homo sapiens chromosome 7 clone RP11-309L24
21	U80460	Human Xq13 3′ end of PAC 92E23 containing the X inactivation transcipt (XIST) gene
26	AL157936	Human DNA sequence from clone RP11-279I21 on chromosome 9q22.2-31.1.
29	X15183	Human mRNA for 90-kDa heat-shock protein
33	AC006016	Homo sapiens PAC clone RP5-968I16 from 7q33-q35
37	AL158075	Human DNA sequence from clone RP11-348K2 on chromosome 9q33.1-34.13
38	AC022022	Homo sapiens chromosome 10 clone RP11-351O1
44	AW954226	EST366296 MAGE resequences, MAGC Homo sapiens cDNA
46	D87684	Homo sapiens mRNA for KIAA0242 protein, partial cds
48	AC004142	Homo sapiens BAC clone CTB-118D7 from 7q31
53	AC013549	Homo sapiens chromosome 11, clone RP11-47J17, complete sequence.
54	D89678	Homo sapiens mRNA for A+U-rich element RNA binding factor
57	AL031778	Human DNA sequence from clone RP -134B21 on chromosome 6p12.1-21.1
63	AA843137	ak06b10.s1 Soares_parathyroid_tumor_NbHPA Homo sapiens cDNA clone IMAGE:1405147 3′ similar to contains Alu repetitive element ;contains element L1 repetitive element
65	BC024242	Homo sapiens, GrpE-like protein cochaperone,clone MGC:33210 IMAGE:4823476, mRNA, complete cds.
66	AC079684	Homo sapiens 12 BAC RP11-351C21 (Roswell Park Cancer Institute Human BAC Library)
76	AV731085	Homo sapiens cDNA clone:HTFAAH04, 5' end,expressed in human hypothalamus.
82	BI036069	IL2-NT0200-030101-325-F09 NT0200 Homo sapiens cDNA, mRNA sequence
97	BU603848	AGENCOURT_8932866 NIH_MGC_141 Homo sapiens cDNA clone IMAGE:6499413 5′, mRNA sequence.

Table 3. Summary of down-regulated genes by MeHg-treatment in human neuroblastoma cells showing accession number and gene name of SSH cDNA clone.

Clone #	Access #	Down-regulation suspected gene name
25	AF104939	Homo sapiens lectomedin-1 gamma (LEC1) mRNA
34	BC000421	Lysosomal-associated protein transmembrane 4
36	BC028182	Chromosome condensation-related SMC-associated protein 1
41	D80004	mRNA for KIAA0182 gene, partial cds
42	AL133511	human DNA sequence from clone RP1-4k15
43	AC019080	BAC clone RP11-337N6 from 2, complete sequence
44	AB088847	BPA-1 mRNA for brain peptide A1, complete cds
45	unknown	unknown
62	BG900849	HOA7-a-D3.T7 HOA (Human Osteoarthritic Cartilage) Homo sapiens cDNA, mRNA sequence
75	unknown	unknown
81	BC039158	Similar to pyruvate dehydrogenase kinase, isoenzyme 1, cds

after treatment with 6.25 μM MeHg. In the case of 6 down-regulated genes (BPA-1: mRNA for brain peptide A1, AL133511: human DNA sequence from clone RP1-4K15, CNAP1: chromosome condensation-related SMC-associated protein 1, LAPTM4A: lysosomal-associated protein transmembrane 4, LEC1: lectomedin-1 gamma, PDK1: pyruvate dehydrogenase kinase, isoenzyme 1), the mRNA expression levels of BPA-1 gene and AL133511 EST were decreased above 2-fold at 6.25 μM and 1 μM MeHg treatment, but the other genes were then only decreased at 6.25 μM MeHg-treated cells.

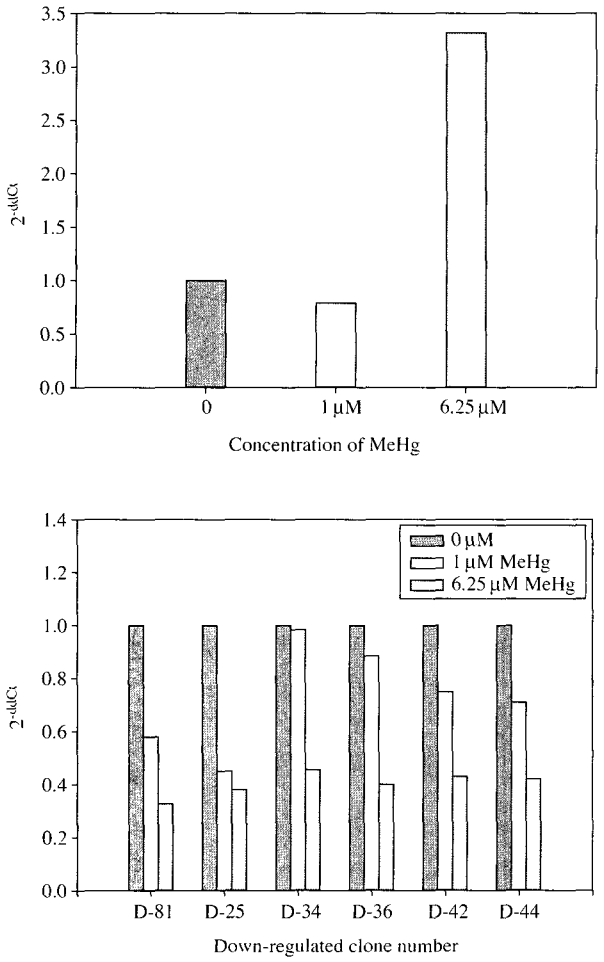

Fig. 3. Relative mRNA levels of positive clone genes for real time RT-PCR verification. The one gene was up-regulated and six genes were down-regulated clones were selected for real time RT-PCR verification through dot blot results. (A) Up-regulated gene. mRNA level of 90 kDa heat shock protein was increased in 6.25 μM MeHg-treated cells. (B) Down-regulated genes. mRNA levels of these six genes were decreased in 6.25 μM MeHg-treated cells. D-44: mRNA for brain peptide A1, D-42: Human DNA sequence from clone RP1-4K15, D-36: chromosome condensation-related SMC-associated protein 1, D-34: lysosomal-associated protein transmembrane 4, D-25: lectomedin-1 gamma, D-81: pyruvate dehydrogenase kinase, isoenzyme 1. Band intensities for seven genes were normalized for GAPDH. Results are expressed as the mean value.

Discussion

The SSH technique was used to identify genes induced by 6.25 μM MeHg in human neuroblastoma SH-SY5Y cells. There is relatively little data on whether or not gene transcription may be modified by cellular MeHg exposure. In this present study, we found increased expression of Hsp90, which has a crucial role in responding to stress and decreased expression 5 genes and 1 EST.

It is well known that heavy metals are able to induce heat shock protein synthesis, thus eliciting a typical cellular stress response[15]. Although metals are known to be effective inducers of stress proteins, the pattern of the induction can vary considerably, depending on the type and dose of administered metal, as well as on metal distribution and accumulation among tissues[15]. In the previous study, it was reported that mercury, cadmium and arsenite enhance Hsp90 synthesis in chick embryos[16]. Also, Brkljacic *et al.*[17] examined that mercury stimulated association of Hsp90 with the glucocorticoid receptor, rendering the cytosolic heat shock protein levels unchanged, suggests that mercury affects the mechanisms controlling the assembly of the receptor heterocomplexes. However, it is few reports that in human neuronal cells, MeHg induce Hsp90 mRNA level. The data support MeHg-induced neurotoxic effects, which are preceded by synthesis of stress proteins, for example Hsp90.

We first identified that mRNA difference of 6 down -regulated genes was associated with MeHg neurotoxicity using the SSH technique. BPA-1 is an unknown function gene and LEC1 is related to G-protein coupled receptor. CNAP1 is down regulated by 1, 25-dihydroxyvitamin D(3) (1,25(OH)(2)D(3)), a potent antiproliferative agent, characterized by a hampered G(1)/S transition[18]. It suggests that inhibitory effects in SH-SY5Y cell proliferation of MeHg may be resulted in the disturbance of cell cycle regulation associated to CNAP1 gene. Also, PDK1 is a key enzyme of glucose oxidation, including the pyruvate dehydrogenase complex (PDC)[19,20]. Nakai *et al.*, mRNA level of PDK isoforms investigated that there was the regional difference in the abundance of mRNAs for PDK isoenzymes in rat brain and that the levels of mRNAs for the isoenzymes were affected by aging[21]. The level of PDK1 mRNA was relatively high in cerebellum and cerebral cortex compared to medulla oblongata and hippocampus of rats. Aging decreased the levels of mRNAs for PDK1 and 2 in cerebellum and increased the PDK2 mRNA in hippocampus and cerebral cortex[21]. So, it suggests that change of energy metabolism by PDK1 down-regulation can induce the neuronal abnormalities in MeHg exposed neuronal system.

Our previous work found that a number of genes responded to MeHg neurotoxic effects[6]. Thus, we hypothesize that the exposure by low and high level of MeHg for short and long term can influence gene transcription in human neuronal cells. The results of this study may be helpful in understanding the phy-

siological and pathological actions of MeHg and could provide clues that MeHg may play an extensive role in gene regulation. Here we report our results for identification of the DEGs responding to MeHg neurotoxicity by using a model of neuroblastoma cells and an improved method for screening DEGs.

In summary, we applied SSH method and real time RT-PCR to identify genes differentially expressed in human neuroblastoma cells as a result of MeHg treatment. This approach resulted in a number of clones composed of both known cDNAs and novel cDNAs. And this analysis resulted in the identification of a number of genes not previously known to be associated with changes in neuronal cells by MeHg. This finding contributes to the understanding of MeHg induced neuronal injury.

Methods

Cell Culture and Treatment

SH-SY5Y cell line purchased from American Type Culture Collection (ATCC) was maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was 90% culture medium (50% F-12 and 50% MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) plus penicillin, streptomycin and non-essential amino acid. The medium was renewed every two days. After reaching to 80% confluency, cells were exposed to various concentrations of MeHg (Aldrich) in culture medium for each exposure time.

MTT Assay

The MTT assay for measuring cytotoxicity and cell growth was performed following the modifications described by Mosmann[22]. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma) is a tetrazolium salt which can be metabolized to formation salt by viable cells and can be quantified spectrophotometrically at 540 nm. Therefore, the MTT assay was used for determination of cell viability.

Isolation and Purification of Total RNA and mRNA

RNeasy Mini Kit and Oligotex Mini mRNA Kit (Qiagen) were used in the isolation and purification of total RNA and mRNA from the cultured human neuroblastoma cells. The protocol was followed according to the manufacturer's specifications.

Establishing Subtracted cDNA Libraries by SSH

Reverse transcription was performed on 2 μg of mRNA obtained from MeHg-treated cells and vehicle-treated cells for 6 hr. After the cDNA was synthesized, SSH was performed using PCR-Select cDNA Subtraction Kit (Clontech), according to manufacturer's protocol. We used cDNAs from 6.25 μM MeHg-treated SH-SY5Y cells as tester and that from DMSO-treated cells as driver for forward subtraction to identify cDNA up-regulated by MeHg treatment, and vice versa for reverse-subtraction to identify cDNA down-regulated in MeHg-treated SH-SY5Y cells. Unsubtracted control for each subtraction was prepared by the same procedures but without the subtractive hybridization step.

Cloning

The forward and reverse subtracted cDNA library was ligated into the pCR-II-TOPO vector (Invitrogen), a TA cloning system, and transformed into One Shot TOP10 competent cells (Invitrogen). The subtracted library was plated on to LB medium agar plates supplemented with 50 μg/mL ampicillin and treated with 40 mg/mL 5-bromo-4-chloro-3-indol-β-D-galactopyranoside (Stratagene) dissolved in dimethylformamide. Agar plates were incubated overnight at 37°C. Positive colonies containing inserts were inoculated in 100 μL LB medium containing ampicillin. Plasmids containing cloned sequences from the subtracted library were purified.

Differential Screening of the Subtracted Library

In order to eliminate false positives, dot blots of 223 individual clones inserted plasmid DNA were prepared and screened using the differential screening kit (Clontech). The transfected cells were harvested in approximately 1 μL of media from each well, which was transferred onto nitrocellulose membrane (Amersham). This membrane was placed on a LB-agar plate which included 100 μg/mL ampicillin and cultured for 12 to 16 hr. Four identical membranes were prepared at the same time. The colonies were denatured by placing the nitrocellulose membrane on 3 MM paper saturated with 0.5 M NaOH, 1.5 M NaCl for 4 min and neutralized with 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl for another 4 min. The nitrocellulose membrane was placed on blotting paper and allowed to air-dry and then was baked for 1 hr at 80°C. The membranes were then hybridized with peroxidase-labeled forward- and reverse-subtracted cDNA probes, and washed with 0.5 × SSC and 2 × SSC. The blots were developed with chemiluminescence hydrogen peroxide substrate (Amersham Pharmacia Biotech Inc.) exposed to X-ray film, and then the differentially expressed clones were selected. Differentially

expressed clones were sequenced from the plasmid using the M13 reverse primer on a ABI 377 sequencer. Nucleic acid homology searches were performed with the BLAST program provided by the National Center for Biotechnology Information (National Institutes of Health).

Validation of Differential Expression of Genes by Real Time RT-PCR

The mRNA levels for the selenoenzymes in SH-SY5Y, human neuroblastoma cells were analyzed by real time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad). The purified total RNAs were reverse-transcribed into cDNAs by using an Omniscript RT kit (Qiagen). The real time RT-PCR was performed by using a SYBR supermix kit (Bio-Rad), and running for 40-45 cycles at 95°C for 20 s and 60°C for 1 min. The PCR efficiency was examined by serially diluting the template cDNA and the melting curve data were collected to check the PCR specificity. Each cDNA sample was triplicated and the corresponding no-RT mRNA sample was included as a negative control. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was included in every plate to avoid sample variations. The mRNA level of each sample for each gene was normalized to that of the GAPDH mRNA. Relative mRNA level was presented as $2^{[(Ct/GAPDH-Ct/gene\ of\ interest)]}$. All data shown were the mean ± SD of three separate experiments. For quantitative real time RT-PCR, primers used were as Table 1.

Reference

1. U.S. Environmental Protection Agency. Mercury Study Report to Congress, Volume V: Health effects of mercury and mercury compounds. EPA-452/R-97-007. Washington, DC: U.S. Environmental Protection Agency; (1997).
2. Choi, B.H. The effects of methylmercury on the developing brain. *Prog. Neurobiol.* **32**, 447-470 (1989).
3. Castoldi, A.F., Coccini, T., Ceccatelli, S. & Manzo, L. Neurotoxicity and molecular effects of methylmercury. *Brain Res. Bull.* **55**, 197-203 (2001).
4. Vogel, D.G., Margolis, R.L. & Mottet, N.K. The effects of methylmercury binding to microtubules. *Toxicol. Appl. Pharmacol.* **80**, 473-486 (1985).
5. Naganuma, A. *et al.* Overexpression of manganese-superoxide dismutase prevents methylmercury toxicity in HeLa cells. *Life Sci.* **62**, 157-161 (1998).
6. Kim, Y.J., Chai, Y.G & Ryu, J.C. Selenoprotein W as molecular target of methylmercury in human neuronal cells is down-regulated by GSH depletion. Biochem. Biophys. *Res. Commun.* **330**(4), 1095-1102 (2005).
7. Liang, P. & Pardee, A.B. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971 (1992).
8. Sokolov, B.P. & Prockop, D.J. A rapid and simple PCR-based method for isolation of cDNAs from differentially expressed genes. *Nucl. Acids Res.* **22**, 4009-4015 (1994).
9. Lisitsyn, N. & Wigler, M. Cloning the differences between two complex genomes. *Science* **259**, 946-951 (1993).
10. Akopian, A.N. & Wood, J.N. Peripheral nervous system-specific genes identified by subtractive cDNA cloning. *J. Biol. Chem.* **270**, 21264-21270 (1995).
11. Deleersnijder, W. *et al.* Isolation of markers for chondro-osteogenic differentiation using cDNA library subtraction. Molecular cloning and characterization of a gene belonging to a novel multigene family of integral membrane proteins. *J. Biol. Chem.* **271**, 19475-19482 (1996).
12. Butte, A. The use and analysis of microarray data. *Nat. Rev. Drug Discov.* **1**, 951-960 (2002).
13. Diatchenko, L. *et al.* Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**, 6025-6030 (1996).
14. Von Stein, O.D., Thies W.G. & Hofmann, M. A high throughput screening for rarely transcribed differentially expressed genes. *Nucl. Acids Res.* **25**, 2598-2602 (1997).
15. Goering, P.L. & Fisher, B.R. Metals and stress proteins. In: Goyer, R.A. & Cherian, M.G. editors. Toxicology of Metals: Biochemical Aspects. Berlin: Springer-Verlag; 229-266 (1995).
16. Papaconstantinou, A.D. *et al.* Mercury, cadmium, and arsenite enhance heat shock protein synthesis in chick embryos prior to embryotoxicity. *Birth Defects Res. B. Dev. Reprod. Toxicol.* **68**(6), 456-464 (2003).
17. Brkljacic, J., Milutinovic, D.V., Dundjerski, J. & Matic, G. Mercury stimulates rat liver glucocorticoid receptor association with Hsp90 and Hsp70. *J. Biochem. Mol. Toxicol.* **18**(5), 257-260 (2004).
18. Verlinden, L. *et al.* Characterization of the condensin component Cnap1 and protein kinase Melk as novel E2F target genes down-regulated by 1,25-dihydroxyvitamin D3. *J. Biol. Chem.* **280**(45), 37319-37330 (2005).
19. Land, J.M, Booth, R.F, Berger, R. & Clark, J.B. Development of mitochondrial energy metabolism in rat brain. *Biochem. J.* **164**(2), 339-348 (1977).
20. Booth, R.F., Patel, T.B. & Clark, J.B. The development of enzymes of energy metabolism in the brain of a precocial (guinea pig) and non-precocial (rat) species. *J. Neurochem.* **34**(1), 17-25 (1980).
21. Nakai, N. *et al.* The abundance of mRNAs for pyruvate dehydrogenase kinase isoenzymes in brain regions of young and aged rats. *Life Sci.* **68**(5), 497-503 (2000).
22. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63 (1983).