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Original Research

Microarray analysis of global gene expression in *Caenorhabditis elegans* exposed to potassium dichromate

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Abstract Hexavalent chromium [Cr (VI)] can be considered as carcinogen heavy metal to human population. Especially, potassium dichromate [$K_2Cr_2O_7$; Cr (VI)] induces DNA damage response and oxidative stress. It also causes inhibition of protective process including DNA repair as well as apoptosis. However, genomic responses to Cr (VI) exposure in *Caenorhabditis elegans* (*C. elegans*) have not been performed yet. As an alternative animal model, *C. elegans* is well known model for genetical studies including mRNA expression (etc.) and also serves as a living biomonitor in ecotoxicological field. Moreover, recognition of chromium biomarkers for the toxicity assessment is of prime importance. In present study, we have investigated the changes of gene expression in *C. elegans* in response to potassium dichromate exposure using microarray consisting of 22K nematode-specific oligonucleotide probes. We have found 28 genes as a Cr (VI) responsive genes that were differentially expressed (>2 fold) following 24 hours exposure to potassium dichromate. In addition, using the comparative toxicogenomics database, we have deduced molecular targets including cyclin B, alpha-B crystalline, G-protein coupled receptor kinase, nucleobindin, U2AF splicing factor, and SR protein (splicing factor) in response to potassium dichromate toxicity in *C. elegans*. In consistency with previous studies in human, it has also found that alteration in expression level of these genes contributes to particular syndromes including cataract, prostatic neoplasms, liver disease and neurotoxicity syndromes. In conclusion, our investigation would be able to present precise route to figure out important biomarkers in response to potassium dichromate in the field of ecotoxicology.

Keywords: Potassium dichromate, *Caenorhabditis elegans*, Microarray, Biomarker, Comparative toxicogenomics database (CTD)

Introduction

Chromium (Cr) compounds have been concerned as an occupational carcinogen for more than a century[1-3] and have shown genotoxicity in a broad variety of experimental model systems[2-4]. Cr (VI) can be passed through the cell membranes and is reduced by the sulfate transport systems inside the cell[5]. The reduced forms of Cr induce the DNA damage during the replication. In addition, the radicals produced during the reduction of Cr (VI) can induce oxidative stress in the cells[5]. Another study has been reported that absorbed Cr (VI) is reduced by cellular reducing agents to Cr (III) via a number of reactive intermediates. Intracellular reduction of Cr (VI) can lead to DNA damage by two potential mechanisms: (a) oxidative DNA damage resulting from reactive intermediates and (b) direct Cr (III)-DNA interactions[6]. Regard to the retrospective study of Cr (VI)-based mortality in China, it has found higher incidences of lung and stomach neoplasms in people living near the Cr-smelting plant, compared with the general population[7]. This finding

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has been approved with evidence on statistical analysis of these data[8,9]. In contrast, epidemiologic study of Cr (VI) exposure in natural environment as well as drinking water has been concluded that Cr did not provide definitive increase in cancer risk or mortality rates[10]. Of health concern, this point raises to attention on gene expression profiling at transcriptome level by Cr (VI) exposure. Therefore, it is necessary to analyze the expression pattern in term of Cr (VI)-mediated genomic responses through microarray approach.

Microarray analysis makes us possible for high throughput screening of altered gene expression. Recently, the microarray has been emerged as a powerful technology due to quantitative comparison of gene expression. Expression change in gene profile after toxicant treatment promotes us to recognize biomarkers in response to individual toxic chemical. Furthermore, profiling of various genes from the microarray also provides better understanding of mechanistic overview of particular toxicants or xenobiotics as well as prediction of their adverse effects in term of environmental toxicogenomics[11]. In addition to the microarray analysis, comparative toxicogenomics database (CTD) also facilitates us for possible explanation on modes of action in which environmental toxic compounds or pollutants render human abnormalities and diseases[12]. The CTD is a potential tool representing interaction between chemical and gene/protein in related with human diseases as well as association of chemical with gene-affected cellular functions. In this database, it integrates scientifically reviewed and concise information on test chemicals, relevant genes and proteins, and their interactions in both vertebrates and invertebrates.

Caenorhabditis elegans (*C. elegans*) is a well known animal model which has been extensively used for toxicological studies and researches. Additionally, measurement of lethality-based concentration and various end-points studies in multi-generations have been also conducted in this model organism[13]. This nematode organism has advantage on exploration of cellular response and risk assessment due to ease at growth onto either definite agar medium or liquid medium and production of several generations within relatively short period of time "quick reproduction"[13]. Importantly, *C. elegans* genome has been completely sequenced as the first multicellular eukaryote. *C. elegans* also exhibits evolutional conservation in amino-acid sequences of many gene products, relative to human proteins. More than one-third of all human genes are known to have a counterpart among *C. elegans* genes. Hence, it gives advantage on genetic research in great detail with simple manipulation in its growth and life cycle. Since there are available in genetic information among the highly conserved genes, this would lead to high probability to apply *C. elegans* for exploiting the toxicant-mediated transcriptional events[14,15]. This might enable us to predict health harmful effects in human.

In our study, we therefore employed *C. elegans* as an alternative animal model to assess Cr (VI) toxicity. The aim of our study was to discover novel bio-indicators for potassium dichromate toxicity and its putative molecular mechanisms. Here, *C. elegans*-specific DNA microarrays were performed to investigate global changes at mRNA level in response to potassium dichromate under 10% lethality concentration (LC_{10}). The microarray-constituted gene expression levels were confirmed by real-time quantitative RT-PCR. Using comparative toxicogenomics database (CTD), this might facilitate us for better understanding of gene-function correlations and the genetic vulnerability among potassium dichromate-responsive genes.

Results and Discussion

Investigation of potassium dichromate toxicity in L1-stage *C. elegans*

Evaluation of potassium dichromate toxicity in L1-stage *C. elegans* was conducted on the basis of lethality. Synchronization of L1-stage *C. elegans* was initially performed to avoid the bias caused by developmental effect on the toxicity test. After 24 hours exposure with various concentrations of potassium dichromate as indicated in Figure 1, *C. elegans* lethality was examined. None observed effective concentration

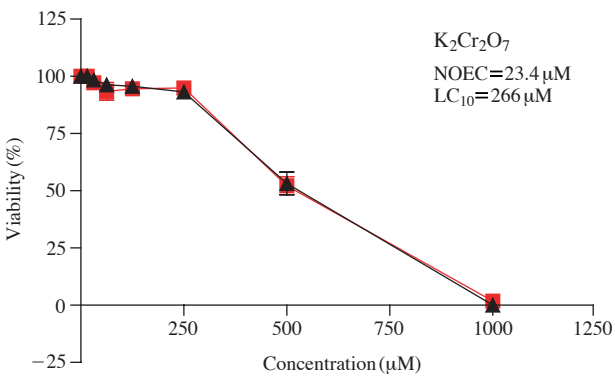


Figure 1. Percentage viability of *C. elegans* to 24 hours potassium dichromate exposure. We determined the NOEC and LC_{10} value as 23.4 μM and 266 μM, respectively. The data point is the means of three independent experiments.

Table 1. 2 fold altered genes in response to potassium dichromate.

Genbank accession	Entrez gene ID	Fold change	Description
Up regulated			
NM_076396	180738	2.90	Nuclear hormone receptor family
NM_064974	191034	2.31	F-box A protein
NM_060656	173092	2.21	RNA-dependent RNA polymerase Family
NM_075720	180408	2.83	Multidrug resistance protein family
NM_182312	353425	3.04	Serpentine receptor, class V
NM_001026585	189771	8.42	UDP-glucuronosyltransferase
NM_001083266	177327	2.08	Metabotropic glutamate receptor family
NM_074279	179993	2.18	Histone H1 Like
NM_182017	172253	3.27	Oxysterol binding protein (OSBP) Related
NM_074859	186004	3.25	C-type lectin
NM_065668	175683	2.12	Kinesin-like protein
NM_072522	192026	20.90	Seven TM receptor
Down regulated			
NM_065692	175703	0.15	Glycyl tRNA synthetase
NM_171880	175983	0.28	Cytokinesis defect
NM_062454	184995	0.08	Toxin-regulated Target of p38MAPK
NM_063454	174394	0.28	Cyclophylin
NM_074218	179967	0.33	RFC (DNA replication factor) family
NM_064461	185408	0.23	Glutathione S-transferase
NM_077610	181374	0.05	Ubiquinol-cytochrome c oxidoreductase complex
NM_065176	175372	0.28	*C.elegans* homeobox
NM_077434	186632	0.48	VEGF (vascular endothelial growth factor) Receptor family
NM_071890	178873	0.21	Heat shock protein
NM_077493	181323	0.13	Acid sphingomyelinase
NM_069090	177675	0.14	Heparan sulphotransferase
NM_064872	186902	0.02	Fatty acid/retinol binding protein
NM_074081	179899	0.12	Metallothionein
NM_001026196	178583	0.20	GEX Interacting protein
NM_070018	178219	0.09	Vacuolar H ATPase

Note: Potassium dichromate toxicity test was carried out at LC_{10} for 24 hours exposure, with statistical significant p-value less than 0.05.

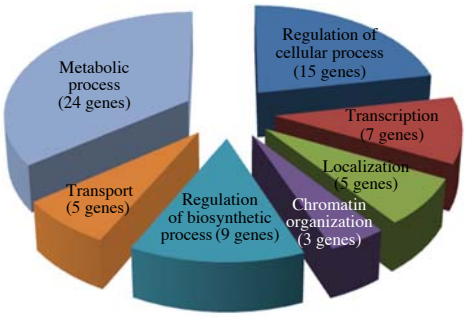
(NOEC) and lethality concentration at 10% (LC_{10}) were determined based on standard criteria[22]. The NOEC and LC_{10} of potassium dichromate in the L1 stage *C. elegans* were determined as 23.4 μM and 266 μM, respectively (Figure 1).

Analysis of gene expression profile upon potassium dichromate via microarray and real-time qRT-PCR approaches

Alteration of genomic expression patterns against potassium dichromate at LC_{10} in synchronized *C. elegans* was analyzed using DNA microarray technology. Twenty eight genes were significantly changed at mRNA level (>2-fold) following potassium dichromate exposure for 24 hours. As notable result (Figure 2), genes involved in regulation of cellular process (15 genes), transcription (7 genes), localization (5 genes), transport (5 genes) and metabolic process (24 genes) were up-regulated in response to potassium dichromate. In addition to the induced-genes, genes involved in cellular homeostasis (19 genes), oxidative phosphorylation (5 genes), cell-cell signaling (16 genes) and secretion (17 genes) were down-regulated. These results might be indicated that potassium dichromate stimulated cellular response in *C. elegans* by affecting on growth-related biological processes, cellular energy production, and homeostatic regulation and stimulating stress-responsive genes in particular pathways.

Interestingly, based on gene ontology (GO) classifications, it was worthy to discover that genes encoding multidrug resistance protein family (MRP) and UDP-glucuronosyltransferase (UGT) were induced toward LC_{10}-potassium dichromate (Table 1). Two fold up-regulated genes MRP and UGT whose products are involved in the transport and metabolic process, respectively (Figure 2A). Moreover, MRP and UGT are also participated in genoprotection and detoxification (Table 1). MRP belongs to ATP binding cassette (ABC) family which has been known to defense against toxic compounds in living organisms[23-25]. De-

(A) 2 fold up-regulated genes (GO analysis)



(B) 2 fold down-regulated genes (GO analysis)

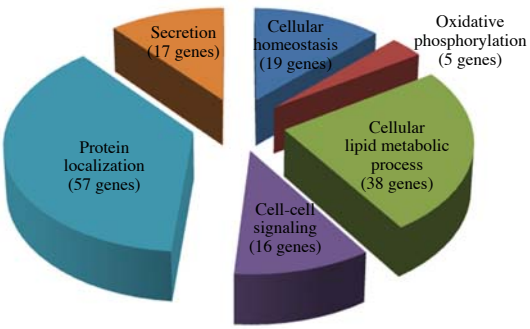


Figure 2. GO analysis of potassium dichromate responsive genes. Among the 2 fold regulated genes, they were classified into various functional groups in response to potassium dichromate. They were divided into 2 fold up-regulated and down-regulated groups.

fense toward toxic substances is a critical biological process in all living organisms[23]. It has been shown that MRP targeted knockout mice were hypersensitive to etoposide, suggesting that MRP assists the organism to protect against toxic compounds[24,25]. Similarly, in *C. elegans*, the deletion mutant of MRP showed more sensitive than wild type *C. elegans* to heavy metals. It has been suggested that MRP might play a part in cellular protective effects in *C. elegans* against heavy metals[26]. In our microarray results, mrp-2 was observed 2 fold up-regulated in response to potassium dichromate. This result might be implied that MRP is up-regulated as cellular response to the potassium dichromate toxicity and plays a protective role against potassium dichromate.

UGT has been reportedly represented as major phase II enzymes of endo- and xenobiotic metabolisms, indicating its role on detoxification and elimination of toxic compounds[27]. Previous study has reported that mutation of UGT1A allele renders the entire locus inactive. It resulted in accumulation of the DNA adducts[28]. Finally, down regulation of UGT was observed in malignant tissue[29]. Particular research focusing on examine the effect of the UGTs in tissues among human populations with diseases intriguingly represents that expression level of UGTs can be considered as one of the criteria to identify for cancer, based on quantitatively analyze differential expression patterns between normal and cancer tissues. Multiple UGT isoforms participate in various metabolisms and elimination of potential direct or indirect human carcinogens, indicating a significant role of this gene on genoprotection[28]. In present work, ugt-18 was observed with 2 fold up-regulation in response to potassium dichromate (Table 1). This result might be implicated that UGT activity is served for detoxifying potassium dichromate toxicity and consequently plays a potential role for cellular protection against potassium dichromate.

Table 2. List of primers used in the real time qRT-PCR.

Gene	Primer sequence
mrp-2	F : 5′-CGG ATG AGG ATC CGA CTT TA-3′
	R : 5′-TGT TGT GGC ACA TAG GCA AT-3′
ugt-18	F : 5′-CCT GCT TAT GGT GCA AGT CA-3′
	R : 5′-CCA GCT CTC TCG TCT CTT CC-3′
gst-6	F : 5′-GCA GGA CAG GAT TAT TCC GA-3′
	R : 5′-ACT CTC TGG CCA AGT ACC GA-3′

Additionally, it was notably observed that genes encoding for glutathione S-transferase (GST) was repressed (Table 1). GST belongs to cellular homeostasis related gene group (Figure 2B). GST has been reported to play a critical role in the protection against ROS. Interference of GST by RNAi in *C. elegans* showed more sensitivity against to 4-hydroxynon-2-enal (4-HNE) which is known as an ROS inducing toxicant[30]. In our microarray data, gst-6 was decreased in response to potassium dichromate exposure, suggesting that perturbing the responsiveness of oxidative stress regulating process in *C. elegans*. It is predicted that potassium dichromate might cause intracellular accumulation of oxidative intermediates and give rise to oxidative stress in *C. elegans*.

Gathering together, these genes mrp-2, ugt-18 and gst-6 might be considered as novel biomarkers for potassium dichromate toxicity in *C. elegans* (Figure 4). Furthermore, real-time qRT-PCR was also carried out to confirm the gene expression pattern from microarray results. Their expression patterns were observed consistent with microarray results (Figure 3).

In silico genomic comparison of potassium dichromate toxicity through comparative toxicogenomics database

Transcriptomic change obtained from the microarray

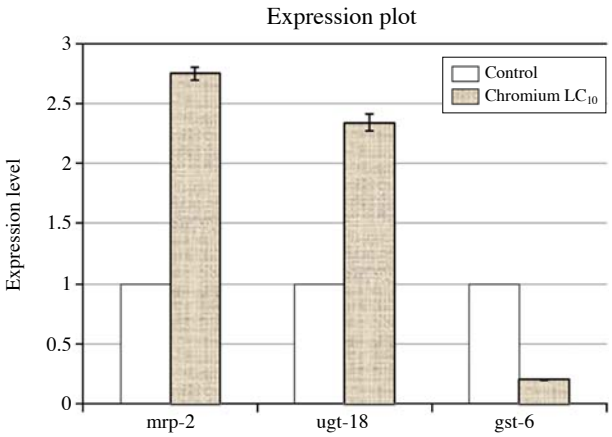


Figure 3. Validation of gene expression pattern using real-time quantitative RT-PCR. Mrp-2, ugt-18, and gst-6 expression levels were confirmed by real-time qRT-PCR. As a result, it was consistent with microarray data.

Table 3. Potassium dichromate responsive genes through the comparative toxicogenomics.

GeneBank ID	Fold change	p-value	Description
NM_069646	2.11	0.10	Cyclin B
NM_066375	0.36	0.05	alpha-B-crystallin
NM_064834	0.25	0.10	G-protein-coupled receptor kinase
NM_171970	0.18	0.00	Nucleobindin homolog
NM_070635	0.10	0.09	U2AF splicing factor
NM_062907	0.10	0.04	SR protein (splicing factor)

analysis was subsequently subjected to toxicogenomics comparison using Comparative Toxicogenomics Database (CTD; http://ctd.mdibl.org/). CTD was utilized to implicate gene expression change between human and *C. elegans* at genomic level[12].

In *C. elegans*-specific microarray results (Table 3), 6 potassium dichromate-related genes whose products are cyclin B, alpha-B crystalline, G-protein coupled receptor kinase, nucleobindin, U2AF splicing factor, and SR protein (splicing factor) were found in consistence with observation of Cr (VI) toxicological study in human hepatoma HepG2 cell line. Among them, alpha-B crystalline, nucleobindin, U2AF splicing factor has been obviously documented to be linked with predictive information focusing on critical syndromes including cataract, prostatic neoplasms, liver disease and neurotoxicity syndromes in the HepG2 cells, respectively. Moreover, the expression level of these genes in response to potassium dichromate at LC_{10} in *C. elegans* exhibited their patterns in agreement with that in the HepG2 cells. These genes could

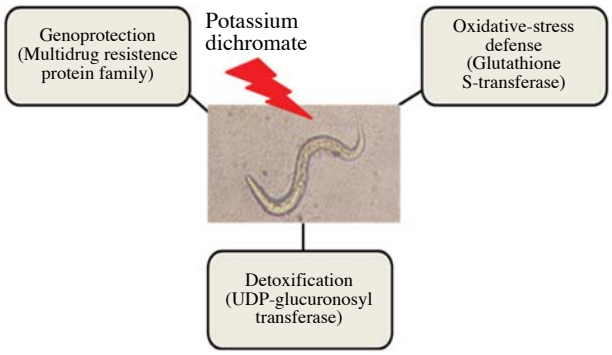


Figure 4. Schematic summary showing potassium dichromate-affected biological responses with novel responsive genes. Mrp-2, ugt-18, and gst-6 are involved in genoprotection, detoxification, and oxidative-stress defense in *C. elegans*, respectively. These discovered genes might be considered as novel bio-markers in response to potassium dichromate in *C. elegans.*

be recognized as novel bio-markers for potassium dichromate risk assessment in human health.

Conclusions

Recently, chromium compound may deleteriously affect several physiological systems including growth, development, and reproduction in the living organisms in the ecosystem. At present study, this is the first demonstration representing the microarray analysis in the *C. elegans* exposed to potassium dichromate. In our study, we identified new potassium dichromate-responsive genes with differential expression including multidrug resistance protein family (mrp-2), UDP-glucuronosyltransferase (ugt-18), and glutathione S-transferase (gst-6) in *C. elegans* due to potassium dichromate exposure. We have intensively studied the potassium dichromate-triggered expression change of individual genes at genomic scale, but the biological consequences of transcriptomic changes caused by this metal are not elucidated yet. In terms of comparative toxicogenomic database, the genomic expression profile of *C. elegans* in response to potassium dichromate might be able to point out bio-monitoring aspects in comparison to human disease predicted-genes. In conclusion, the results from microarray analysis in *C. elegans* exposed to potassium dichromate will assist the researchers to understand genomic responses including genoprotection, detoxification, and oxidative stress defense in higher organisms. However, further research would be required to study the biological consequences of the genes, which would provide detailed information of the newly discovered biomarkers in the field of ecotoxicology.

Materials and Methods

Maintenance of *Caenorhabditis elegans*

N2 strain of *C. elegans* was employed for toxicity test regarding to potassium dichromate. *C. elegans* were cultured on nematode growth media (NGM) plates along with *Escherichia coli* strain DH5α as a food source at 20˚C. N2 strain was gained from Prof. Arizono laboratory of Kumamoto University in Japan.

Potassium dichromate toxicity

In order to produce large amount of population of age-synchronized *C. elegans*, we grew N2 strain *C. elegans* using S-medium in shaking incubator at 20˚C for 72 hours. They were washed using distilled water 2 times and treated with hypochlorite to release egg from the adult body. The eggs were then washed using K-medium 2 times and cultured in K-medium without DH5α as a food source for 16 hours at 20˚C to reach L1-stage. Total 8 concentrations of potassium dichromate (0, 15.7, 31.25, 62.5, 125, 250, 500, and 1,000 μM) were conducted for the lethality test. Counting for number of lethal warm was performed after 24 hours exposure.

Preparation of total RNA for microarray experiment

To isolate total RNA, frozen nematode pellets were ground into fine powder using a liquid nitrogen-chilled mortar and pestle before homogenization in lysis buffer containing 2-mercaptoethanol (provided by Quiagen kit), as previously described[16]. Total RNA was subsequently purified using RNeasy kits (Quiagen, Valencia, CA, USA) prior to microarray experiments[17].

C. elegans whole genome DNA microarray

The microarray experiment was performed in order to study the gene expression affected by potassium dichromate at LC_{10}. In this study, the 22K oligo chip (Washington University) was used. RNA samples from potassium dichromate-treated and non-treated *C. elegans* were amplified and microarray experiment process was carried out with Amino Allyl MessageAmp aRNA Kit (Ambion Co.) in according to the manufacture's protocol. DNA chips were scanned using GenePix 4000B (Axon Instruments, Union City, CA) and then were analyzed with GenePix Pro 3.0 software (Axon Instruments, Union City, CA) to obtain gene expression ratios. Logged gene expression ratios were normalized by lowess regression[18].

Analysis of microarray expression data

Subio platform ver. 1.6 was used for the initial analysis of expression data. Lowess normalization was applied before further data processing. Expression changes are described by fold change (expression ratio between treated and control signals)[19]. Identification of potassium dichromate responsive genes through the comparative toxicogenomics database was performed to study potassium dichromate affected gene expression and potassium dichromate related disease.

Quantitative real time RT-PCR

qRT-PCR was performed using Power SYBR Green PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Act-1 was used as a control to normalize transcript levels[20]. PCR primers designed by Primer 3[21] which is denoted in Table 2. The real-time PCR analysis was undertaken on an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA).

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