



*Toxicomics Report*

## Knockdown of deubiquitinating enzyme Usp34 confers resistance to methylmercury in HEK293 cells

Jong-Mu Kim<sup>1</sup>, Jin-Yong Lee<sup>1,2</sup>, Min-Seok Kim<sup>1,3</sup>, Sawako Shindo<sup>4</sup>, Takeshi Kumagai<sup>4</sup>, Akira Naganuma<sup>1</sup> and Gi-Wook Hwang<sup>1,4</sup>

<sup>1</sup>Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Miyagi 980-8578, Japan

<sup>2</sup>Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan

<sup>3</sup>Inhalation Toxicology Research Group, Korea Institute of Toxicology, 30, Baekhak1-gil, Jeongeup-si, Jeollabuk-do, 56212, Republic of Korea

<sup>4</sup>Laboratory of Environmental and Health Sciences, Faculty of Pharmaceutical Sciences, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, Japan

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**ABSTRACT** — We have previously reported that the ubiquitin–proteasome system, which is a selective proteolytic system, plays an important role in determining sensitivity to methylmercury in various cultured cells. Deubiquitinating enzyme is a negative regulator of protein degradation through the ubiquitin–proteasome system. In the present study, we searched for deubiquitinating enzymes that affect sensitivity to methylmercury by RNA interference, and identified ubiquitin-specific protease 34 (Usp34) as a deubiquitinating enzyme that confers methylmercury resistance to HEK293 cells by suppressing gene expression.

**Key words:** Methylmercury, Sensitivity, Deubiquitinating enzyme, Ubiquitin-specific protease 34 (Usp34), RNA interference

### INTRODUCTION

Methylmercury is an environmental pollutant that is selectively toxic to the central nervous system (Carocci *et al.*, 2014; Diez, 2009; Grandjean *et al.*, 1997; Tatsuta *et al.*, 2017). More than 60 years have passed since the discovery of Minamata disease after a large population was afflicted with the disease after ingestion of large amounts of methylmercury (Al-Ardhi and Al-Ani, 2008; Sheehan *et al.*, 2014), but the mechanisms involved in its toxicity remain unclear. We have previously reported that there are many proteins involved in methylmercury toxicity and that the intracellular levels of some of them are regulated by the ubiquitin–proteasome (UP) system (Hwang *et al.*, 2002; Lee *et al.*, 2020; Naganuma *et*

*al.*, 2002; Toyama *et al.*, 2021, 2020). Therefore, the UP system might play an important role in determining the sensitivity of cells to methylmercury toxicity. Additionally, deubiquitinating enzyme negatively regulates cellular responses through ubiquitination. Deubiquitinating enzyme catalyzes degradation of polyubiquitin chains and removal of ubiquitin molecules from ubiquitinated proteins by hydrolyzing and cleaving the C-terminal peptide or isopeptide bonds of ubiquitin (Wilkinson, 2000). Therefore, deubiquitinating enzyme has attracted attention as a regulatory factor that inhibits various cellular responses induced by ubiquitination, such as proteolysis and signal transduction (Hanpude *et al.*, 2015). However, the relationship between methylmercury toxicity and deubiquitinating enzyme has not been investigated. In

Correspondence: Gi-Wook Hwang (E-mail: [hwang@tohoku-mpu.ac.jp](mailto:hwang@tohoku-mpu.ac.jp))

this study, we searched for deubiquitinating enzymes that affect the sensitivity of cells to methylmercury by suppressing gene expression through RNA interference.

## MATERIALS AND METHODS

### Cell line and culture condition

Human embryonic kidney 293 (HEK293) cells were used to search for deubiquitinating enzymes that confer methylmercury resistance by siRNA transfection. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C.

### Simple screening for deubiquitinating enzyme involved in methylmercury toxicity

Silencer™ Negative Control siRNA and double-stranded siRNAs, each of which recognized approximately 60 species of deubiquitinating enzyme, were purchased from Ambion (Austin, TX, USA). Transfection of each siRNA into HEK293 cells was performed using HiPerFect® transfection reagent (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Briefly, 2.5 µL of double-stranded siRNA (10 nM final concentration) was added to each well of a 96-well plate. Then, 0.75 µL HiPerFect® transfection reagent was diluted in 24.25 µL of Opti-MEM (Invitrogen, Carlsbad, CA, USA), and the diluted reagent was added to the wells. The plates were incubated at room temperature for 15 min to form transfection complexes. Then, 65 µL of cell suspension with  $1 \times 10^4$  cells was added to the wells. After 24 hr of incubation, 10 µL methylmercuric chloride (5 µM final concentration) at a concentration sufficient to inhibit normal cell growth by approximately 50% was added to the cells, followed by an additional 48 hr of culture. Cell viability was monitored using alamarBlue® (Invitrogen). Additionally, screening was performed using two types of siRNAs with different sequences for each gene, which was evaluated by calculating the average of the survival rates.

### Quantitative real-time PCR

The target sequences that corresponded to USP34 mRNA were as follows: siRNA #1, GGATCTAGCAATGAGGTTA; siRNA #2, GATCTTAGGGCTGAAGTAA. siRNA-transfected cells (approximately  $1 \times 10^6$  cells/well in 6-well plates) were incubated for 24 hr and then washed with PBS. Total RNA was isolated using Isogen II (Nippon Gene, Toyama, Japan). First-strand cDNA synthesis was performed using a PrimeScrip® RT reagent kit (Takara, Shiga, Japan) in accordance

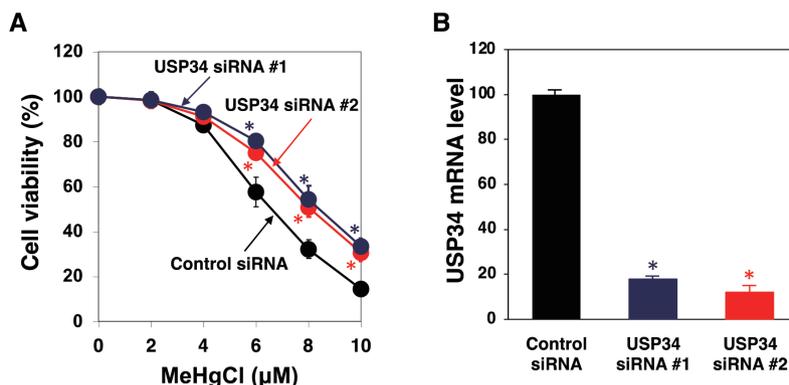
with the manufacturer's instructions. Quantitative real-time PCR of the USP34 gene was performed using sense, 5'-AAGAGCGCCACAAGCTATT-3', antisense, 5'-ACTTCCCGTAGACTCCCTCC-3' and that of the GAPDH gene was performed using sense, 5'-GCACCGTCAAGGCTGAGAAC-3', antisense, 5'-TGGTGAAGACGCCAGTGGA-3'. The USP34 mRNA levels were determined by a standard curve after calibration of the assay.

## RESULTS AND DISCUSSION

It is known that approximately 100 species of deubiquitinating enzymes exist in humans (Hanpude *et al.*, 2015). We transfected HEK293 cells with siRNA (Ambion) that recognized genes encoding 60 species of these enzymes for 24 hr. Then, these cells were treated with methylmercuric chloride at 5 µM, a concentration that inhibited cell proliferation by approximately 50%, for 48 hr. Usp33, Usp34, and Usp47 were selected as candidate deubiquitinating enzymes that conferred methylmercury resistance to HEK293 cells by gene knockdown. We next re-examined whether methylmercury resistance was acquired by suppressing the expression of these genes using siRNA from Sigma-Aldrich. When cells transfected with two types of double-stranded siRNAs, each of which had a different sequence that recognized these genes, were treated with various concentrations of methylmercury, cells with suppressed expression of Usp34 showed resistance to methylmercury compared with control cells (Fig. 1A). The USP34 mRNA levels were decreased by more than 80% in siRNA-transfected cells compared with control cells (Fig. 1B). Cells with suppressed Usp33 or Usp47 expression did not show methylmercury resistance and had the same level of methylmercury sensitivity as control cells (data not shown). These results suggest that Usp34 is a novel factor involved in the enhancement of methylmercury toxicity.

Usp34 negatively regulates ubiquitination of gp78 and Sox2, thereby inhibiting their degradation in the proteasome (Dai *et al.*, 2020; Wang *et al.*, 2019). gp78 is a ubiquitin ligase involved in endoplasmic reticulum-associated degradation, which is protective against endoplasmic reticulum stress (Shen *et al.*, 2006; Zhong *et al.*, 2004). However, the contribution of endoplasmic reticulum stress to enhancement of methylmercury toxicity by Usp34 appears to be low, because degradation of gp78 by the UP system may be promoted and its levels were decreased in Usp34 knockdown cells. Sox2 is also unlikely to be involved in the enhancement of methylmercury toxicity by Usp34, because Sox2 promotes cell prolifer-

## Usp34 enhances methylmercury toxicity in HEK293 cells



**Fig. 1.** Effects of Usp34 knockdown on sensitivity to methylmercury in HEK293 cells. (A) HEK293 cells ( $1 \times 10^4$  cells/well) transfected with two different siRNAs against USP34 mRNA were seeded in 96-well plates and cultured in 100  $\mu$ L aliquots of medium. After transfection with siRNA for 24 hr, various concentrations of methylmercuric chloride were added and the cells were cultured for an additional 48 hr. Cell viability was measured by the alamarBlue<sup>®</sup> assay. Each point and bar represents the mean value and standard deviation of the results from three cultures, respectively. \*  $p < 0.01$  vs. control siRNA. (B) USP34 mRNA levels in HEK293 cells transfected with two different siRNAs against USP34 mRNA were analyzed by quantitative real-time PCR. USP34 mRNA levels were normalized to GAPDH mRNA. \*  $p < 0.01$  vs. control siRNA.

ation. It was recently reported that Usp34 promotes cell proliferation through activation of PKC and AKT signaling pathways (Gu *et al.*, 2019). Although Usp34 is involved in the promotion of cell proliferation and inhibition of cell death in various cell lines, the present study suggests that Usp34 is also involved in the enhancement of methylmercury toxicity. Usp34 may inhibit degradation of proteins involved in the enhancement of methylmercury toxicity through the UP system. Therefore, identification of proteins involved in methylmercury toxicity among substrates recognized by Usp34 is expected to clarify the mechanism of methylmercury toxicity.

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**Conflict of interest**---- The authors declare that there is no conflict of interest.

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