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Arsenic (+3 oxidation state) methyltransferase is a specific but replaceable factor against arsenic toxicity

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ABSTRACT

Inorganic metalloids, such as arsenic (As), antimony (Sb), selenium (Se), and tellurium (Te), are methylated in biota. In particular, As, Se, and Te are methylated and excreted in urine. The biomethylation is thought to be a means to detoxify the metalloids. The methylation of As is catalyzed by arsenic (+3 oxidation state) methyltransferase (AS3MT). However, it is still unclear whether AS3MT catalyzes the methylation of the other metalloids. It is also unclear whether other factors catalyze the As methylation instead of AS3MT. Recombinant human AS3MT (rhAS3MT) was prepared and used in the *in vitro* methylation of As, Se, and Te. As, but not Se and Te, was specifically methylated in the presence of rhAS3MT. Then, siRNA targeting AS3MT was introduced into human hepatocarcinoma (HepG2) cells. Although AS3MT protein expression was completely silenced by the gene knockdown, no increase in As toxicity was found in the HepG2 cells transfected with AS3MT-targeting siRNA. We conclude that AS3MT catalyzes the methylation of As and not other biomethylatable metalloids, such as Se and Te. We speculate that other methylation enzyme(s) also catalyze the methylation of As in HepG2 cells.
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1. Introduction

A metalloid is an element that has properties in between those of metals and non-metals. This unique characteristic makes metalloids a valuable material in industry. In particular, arsenic (As), antimony (Sb), selenium (Se), and tellurium (Te) have found various uses. In the periodic table, As and Sb belong to group 15, and Se and Te belong to group 16. In addition, As and Se are in the fourth period, and Sb and Te are in the fifth period. Enzymes that require Se in the form of selenocysteine are known; thus, Se is an essential element in animals. Enzymes that require As, Sb, and Te have not been identified so far.

As is used as a wood preservative, a pesticide, a glass clarifying agent, and a material for semiconductors. In addition, arsenic trioxide is used as a therapeutic agent for acute promyelocytic leukemia [1]. On the other hand, inorganic As compounds are carcinogenic, and As environmental pollution has attracted attention worldwide [2]. Sb is a rare metal that is used as a flame retardant in textile, an ingredient in paint, and a component of batteries. Like As, inorganic Sb compounds are more hazardous than organic Sb compounds. Antimony trioxide is classified in group 2B

Abbreviations: AS3MT, arsenic (+3 oxidation state) methyltransferase; $MMAs^{V}$, monomethylarsonic acid; ICP–MS, inductively coupled plasma–mass spectrometry; $DMAs^{V}$, dimethylarsinic acid; SAM, *S*-adenosyl-L-methionine; TMSe, trimethylselenonium ion; TMTe, trimethyltelluronium ion.

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by the International Agency for Research on Cancer (IARC) [3]. Se is also widely used in industry, and is an essential trace element in human. Se deficiency causes such health problems as myocardial necrosis [4]. Meanwhile, the ingestion of excess amounts of Se causes Se toxicosis, which is characterized by vomiting, diarrhea, and muscle cramps [5]. Te is a rare metal that is used in phase-change digital versatile discs, solar panels, and Peltier devices. As the clinical symptoms of Te toxicity, garlic breath, vomiting, and pigmentation of the oral mucosa are reported [6]. Although those metalloids are indispensable for human life, they may pose serious problems to human health and the environment.

In animals, inorganic As is primarily transformed into methylated metabolites, such as monomethylarsonic acid ($MMAs^{V}$) and dimethylarsinic acid ($DMAs^{V}$). The enzyme catalyzing the methylation is arsenic (+3 oxidation state) methyltransferase (AS3MT) [7]. Sb, which belongs to the same group as As, is methylated not in animals but in microorganisms [8]. Sb is excreted in urine as coordinated compounds with organic anions, such as citrate [9]. In contrast, Se and Te are biomethylatable species in animals. Inorganic Se ingested in human and animals is primarily excreted in urine as selenosugars, *e.g.*, Se-methylseleno-*N*-acetylgalactosamine [10,11]. Urinary selenosugars are methylated compounds. In addition to selenosugars, trimethylselenonium ion (TMSe) is also a urinary Se metabolite [12]. TMSe is excreted in urine when the amount of Se ingested exceeds the nutritional level [13]. Te is also excreted in urine as trimethyltelluronium ion (TMTe) [14,15]. Interestingly, no Te-containing sugars, tellurosugar, have been identified in urine so far. The mechanisms underlying the methylation of the group 16 metalloids, *i.e.*, Se and Te, are still unclear. Recombinant AS3MTs was prepared from rat [16], human [17,18] and alga [19] genes, and their function and enzymatic characteristics were evaluated. However, the involvement of AS3MT in the biomethylation of other metalloids has not been evaluated, yet. Hence, the aim of this study is to examine whether AS3MT can catalyze the methylation of Se and Te or not. We also evaluate whether AS3MT is a specific factor for As methylation or not. Then, we attempt to clarify the characteristics of AS3MT in the metalloid methylation.

2. Materials and methods

2.1. Reagents

Phosphate-buffered saline (PBS) (−) and primers for PCR were purchased from Life Technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA), Trizma® base (tris(hydroxymethyl)aminomethane), L-glutathione reduced form (GSH), and catalase were purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Mercaptoethanol (β-ME) and *S*-adenosyl-L-methionine (SAM) were purchased from Nacalai Tesque (Kyoto, Japan) and Zymo Research (Irvine, CA, USA), respectively. Bacto Tryptone, Bacto Yeast Extract, and Bacto Agar were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). TMSe was purchased from Tri Chemical Laboratories (Yamanashi, Japan). TMTe was synthesized in our laboratory [20]. Monomethyltellurous acid was provided by Otsuka Chemical Co., Ltd. (Tokyo, Japan). Sodium arsenite (iAs^{III}), disodium hydrogen arsenate heptahydrate (iAs^{V}), $DMAs^{V}$, sodium tellurite (iTe^{IV}), sodium tellurate (iTe^{VI}), and other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell culture

Human hepatocarcinoma cells, HepG2 cells, were obtained from Riken BioResource Center (Tsukuba, Japan). The HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (FBS; Hana-Nesco Bio, Tokyo, Japan) and 100 U/mL penicillin plus 100 μg/mL streptomycin (Life Technologies) at 37 °C under a humid atmosphere of 5% CO_2.

2.3. Preparation of histidine-tagged recombinant human AS3MT (rhAS3MT)

Total RNA was extracted from the HepG2 cells by means of a ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA), and cDNA was prepared from 2 μg of the total RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). AS3MT cDNA lacking the termination codon was amplified by PCR with TaKaRa Ex Taq (1000 U) (Takara Bio, Shiga, Japan) and 10 μM primers (sense: 5′-ATGGCTGCACTTCGTGAC-3′, antisense: 5′-GCAGCTTTTCTTTGTGCCAC-3′). The PCR conditions were as follows: denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 7 min. The PCR product was inserted into pBAD vector located downstream of the arabinose-responsive element, and fused with six repeated histidine (His) elements for tagging using a pBAD TOPO TA Expression Kit (Life Technologies) for 10 min at room temperature. pBAD-inserted rhAS3MT (pBAD-AS3MT) was cloned in competent *Escherichia coli*, DH10B, according to the manufacturer's instructions. The DH10B cells were cultured in SOC medium (Life Technologies) at 37 °C with gentle shaking at 190 rpm for 1 h, and then, the transformed DH10B cells were selected in Luria-Bertani (LB) agar medium containing 1% Bacto Tryptone, 0.5% Yeast Extract, 1% NaCl, and 1.5% Bacto Agar with 0.1 mg/mL ampicillin (Wako Pure Chemical Industries) at 37 °C overnight.

DH10B cells transformed with pBAD-rhAsS3MT were pre-incubated in 5 mL of LB medium containing 0.1 mg/mL ampicillin at 37 °C overnight with gentle shaking at 190 rpm. Then, a 5 mL aliquot of the cell suspension was cultured in 500 mL of LB medium containing 0.1 mg/mL ampicillin at 37 °C for 2.5 h with gentle shaking at 190 rpm. After the incubation, 2% L-(+)-arabinose (Sigma-Aldrich) was added to induce rhAsS3MT protein for 4 h. Then, the cells were collected and homogenized by sonication. The homogenate was centrifuged at 39,000 × *g* for 20 min. His-tagged rhAS3MT protein in the supernatant was purified by a nickel affinity column (His GraviTrap, GE Healthcare). The eluate was dialyzed with Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific, Waltham, MA,

Table 1
HPLC conditions for the separation of metalloid metabolites catalyzed by rhAS3MT.

Metalloid	As	Se and Sb	Te
Column	PRP X-100	GS-320HQ	ES-502N 7C
(Hamilton; Reno, NV, USA)	(Shodex; Tokyo, Japan)	(Shodex; Tokyo, Japan)	
Separation mode	Anion exchange	Multi-mode size exclusion	Anion exchange
Column size (mm)	4.6 i.d. × 150 7.5 i.d. × 300	7.5 × 150	
Flow rate (mL/min)	0.8	0.6	1.0
Eluent	50 mM ammonium acetate (pH 6.5)	←	15 mM citric acid (pH 2.0)

USA). The quality and quantity of rhAS3MT were evaluated by Western blotting, as discussed below.

2.4. In vitro methylation of metalloids and speciation analysis of methylated metabolites by LC–ICP–MS

The assay mixture consisted of 7 mM GSH, 1 mM SAM as the methyl group donor, 1 μM metalloid, such as iAs^{III}, iSe^{IV}, iSb^{III}, or iTe^{IV}, and 30 μg of rhAS3MT in 20 mM sodium phosphate buffer. The assay mixture was incubated at 37 °C for 4 h, and then heated at 95 °C for 5 min to terminate the reaction. H_2O_2 was added to the mixture at the final concentration of 3%, and the mixture was further incubated for 1 h. Then, 50-fold diluted catalase was added to eliminate excess H_2O_2 from the mixture. The mixture was centrifuged at 14,000 × g for 30 min, and filtered through a 0.45-μm membrane filter. A 50 μL aliquot of the filtrate was applied to an HPLC (Prominence, Shimadzu, Kyoto, Japan) coupled with an inductively coupled plasma mass spectrometer (ICP–MS; 7500ce, Agilent Technologies, Hachioji, Japan). The columns used for the separation of each metalloid are shown in Table 1. As, Se, Sb, and Te in the eluate were detected by ICP–MS at *m/z* 75, 82, 121, and 130, respectively.

2.5. AS3MT gene knockdown by siRNA

HepG2 cells were plated at 3 × 10 cells/cm^2 and cultured for 24 h. siRNA targeting AS3MT or control siRNA was introduced into the HepG2 cells at the final concentration of 100 nM with Lipofectamine RNAiMAX (final concentration: 0.3%; Life Technologies), and the cells were incubated in Opti-MEM I (Life Technologies) for 20 min at room temperature. The siRNA targeting AS3MT and the control siRNA were as follows: AS3MT siRNA, 5′-GAAGUAGCCCUAAGAUAUUTT-3′; and control siRNA, 5′-UUCCGUCUCGAAAGAGUUUAGUCCU-3′.

2.6. Real-time RT–PCR

Total RNA was extracted and cDNA was synthesized by the same methods as those mentioned above from siRNA-treated HepG2 cells. Real-time PCR was carried out using Power SYBR Green Master Mix (Life Technologies) on an Applied Biosystems StepOne Real-Time PCR System (Life Technologies) according to the manufacturer's instructions. Specific primers for AS3MT (sense, 5′-GCCTTGCAAAATGTACACGAA-3′; antisense, 5′-TGCCACTTCCACTACCCAGA-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as control (sense, 5′-GCACCGTCAAGGCTGAGAAC-3′; antisense, 5′-TGGTGAAGACGCCAGTGGA-3′) were used.

2.7. Western blot analysis

HepG2 cells treated with each siRNA were washed with ice-cold PBS(−) and collected with 50 mM Tris–HCl (pH 7.4). The pellets were homogenized by sonication for 15 min, and then centrifuged at 105,000 × g for 1 h. A 20 μg portion of protein in the supernatant was separated by SDS-PAGE, and then transferred onto polyvinylidene fluoride membrane (Hybond-P, GE Healthcare) at 20 V for 1 h. The membrane was blocked for 1 h with 3% BSA in PBS(−) containing 0.1% Tween-20 (PBS-T). For the detection of AS3MT, the membrane was washed briefly with PBS-T and incubated with anti-Cyt19 (AS3MT) rabbit polyclonal antibody (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 100-fold with PBS-T containing 5% BSA overnight at 4 °C. The membrane was washed with PBS-T, and probed with horseradish-peroxidase-conjugated anti-rabbit IgG (1:50,000) (GE Healthcare). The bands were visualized with Immobilon Western Chemiluminescent Substrate (Merck Millipore, Billerica, MA, USA) and LAS-1000 UV mini (FUJIFILM, Tokyo, Japan). For the detection of rhAS3MT, anti-His-tag antibody (GE Healthcare) was also used as the primary antibody in addition to anti-Cyt19 antibody. Anti-GAPDH mouse polyclonal antibody (Santa Cruz Biotechnology) and horseradish-peroxidase-conjugated anti-mouse IgG (GE Healthcare) were used for the detection of GAPDH.

2.8. Assay for cytotoxicity

HepG2 cells were seeded in a 96-well plate. HepG2 cells treated with AS3MT or control siRNA were treated with 0.1, 0.5, 1.0, 5, 10, 50, 100, 500, and 1000 μM iAs^{III}, iSe^{IV} or iTe^{IV} in FBS-free medium for 24 h. After the incubation, the medium was discarded and the cells were washed twice with PBS(−). The cells were subjected to CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) for 4 h, and cell viability was determined by measuring the absorbance at 490 nm.

2.9. Statistical analysis

Data are presented as means ± standard deviation (S.D.) of three to four independent experiments. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni-type multiple *t*-test. Differences between the groups were significant at $p < 0.05$.

3. Results and discussion

3.1. In vitro methylation of metalloids

The quantity and quality of rhAS3MT were examined by Western blotting (data not shown). As the quantity and quality were sufficient, rhAS3MT was used for the latter experiments. rhAS3MT catalyzed the methylation of As, which was proven by the appearance of novel peaks of As at the retention times of 3.4 and 3.8 min (Fig. 1B). That the retention time of the former peak corresponded to that of $DMAs^{V}$ standard suggested that rhAS3MT was able catalyze the methylation of arsenite in the presence of GSH and SAM (Fig. 1B and C). Although monomethylarsonic acid ($MMAs^{V}$) standard was not available, the latter peak seemed to be $MMAs^{V}$, a metabolic intermediate.

Only inorganic Se compounds, such as selenite (iSe^{IV}) and selenate (iSe^{VI}), were detected at the retention times of 17.0 and 14.4 min, respectively, even in the presence of rhAS3MT. TMSe, a urinary metabolite of Se, was not detected (Fig. 2). Similar results were obtained for Te, namely, no methylated metabolites, such as TMTe and MMTe, were detected even in the presence of rhAS3MT (Fig. 3B). Two minor peaks were detected at the retention times of 2.1 and 2.4 min in the presence and absence of AS3MT (Fig. 3A and 3B). Because the peaks were detected even in the absence of AS3MT, it was speculated that those Te species were complexes with GSH or SAM. It is known that Se and Te are also methylated *in vivo* [14,21]. However, our results suggest that AS3MT does not catalyze the methylation of Se and Te. We also found that rhAS3MT did not catalyze the methylation of iSb^{III} *in vitro* (data not shown). This observation is coincident with the fact that iSb^{III} is not methylated *in vivo* [22]. Consequently, although inorganic As, Se, and Te are methylated *in vivo*, AS3MT is specific to As. In bacteria, the methylation of Se and Te is catalyzed by thiopurine methyltransferase (TPMT) [23]. TPMT is also found in mammals. Hence, although we were unable to present direct evidence in this study, TPMT could be the responsible methyltransferase that catalyzes the methylation of Se and Te in mammals.

3.2. Effect of AS3MT gene knockdown on cytotoxicity of metalloids

The treatment with siRNA targeting AS3MT reduced AS3MT mRNA expression to around 30% of control (Fig. 4A), and no apparent expression of AS3MT protein was detected by Western blotting (Fig. 4B). Hence, sufficient effects of siRNA could be obtained under the experimental conditions adopted in this study.

The viability of HepG2 cells transfected with AS3MT-targeting siRNA was not significantly different from that of cells transfected with control siRNA (Fig. 5). It is reasonable that no differences are observed in the cell viability between HepG2 cells transfected with AS3MT-targeting and control siRNA against the Se and Te exposure, because AS3MT does not contribute to the detoxification, *i.e.*, the methylation of Se and Te, as shown in Figs. 2 and 3. In contrast, As was substantially methylated in the presence of AS3MT *in vitro*. However, the methylation catalyzed by AS3MT did not contribute to the detoxification of As. Those results lead us to two speculations. First, the methylation is not a detoxification process but a metabolic activation of As

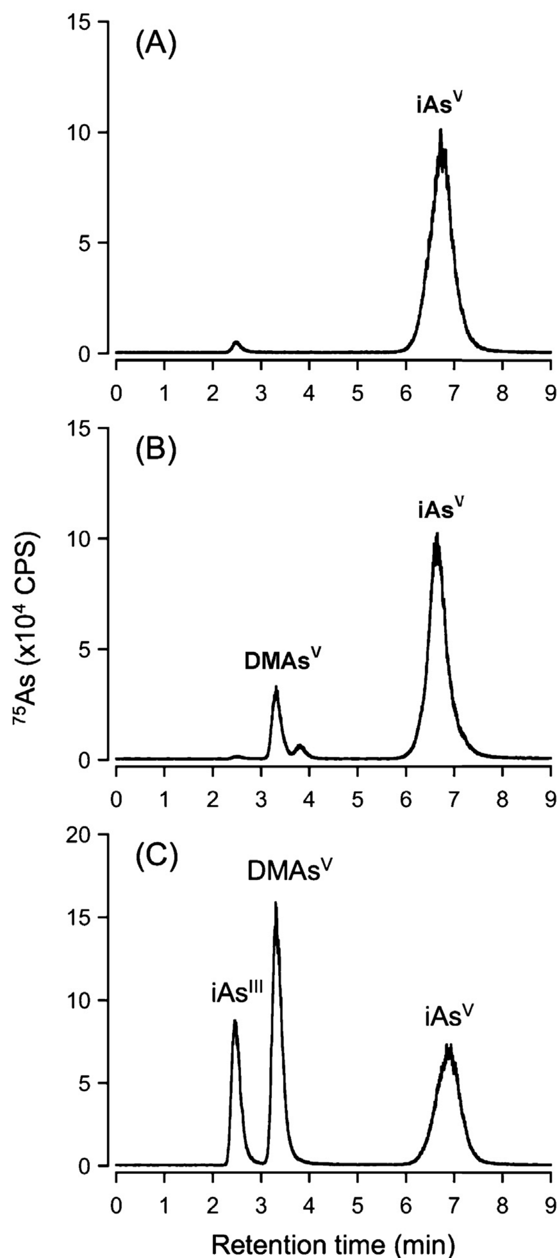

Fig. 1. Elution profile of As in the assay mixture. The assay mixture consisted of 7 mM GSH, 1 mM SAM as the methyl group donor, and 1 μM iAs^{III} in 20 mM sodium phosphate buffer in the presence (B) or absence (A) of 30 μg of rhAS3MT. The assay mixture was incubated at 37 °C for 4 h, and then heated at 95 °C for 5 min to terminate the reaction. H_2O_2 was added to the mixture at the final concentration of 3%, and further incubation was carried out for 1 h. Then, 50-fold diluted catalase was added to eliminate excess H_2O_2 from the mixture. A 50 μL aliquot of the filtrate was applied to PRP X-100, an anion exchange column. The eluate was directly introduced to an ICP–MS to detect As at *m/z* 75. As standards, such as iAs^{III}, iAs^{V}, and $DMAs^{V}$ without treatment with H_2O_2, were also analyzed (C).

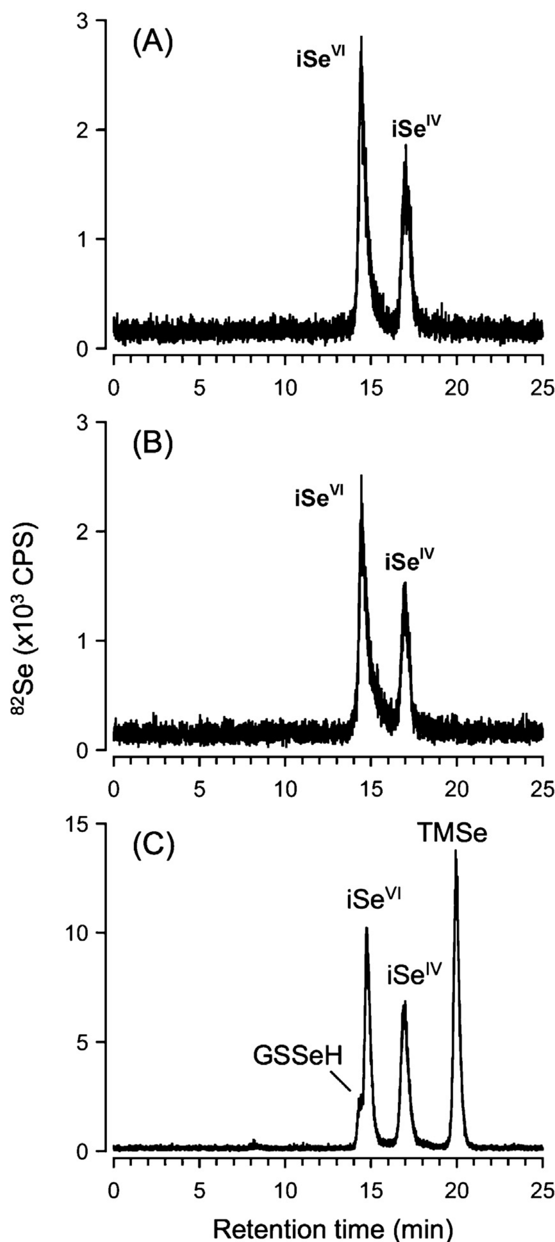

Fig. 2. Elution profile of Se in the assay mixture. The assay mixture consisted of 7 mM GSH, 1 mM SAM as the methyl group donor, and 1 μM iSe^{IV} in 20 mM sodium phosphate buffer in the presence (B) or absence (A) of 30 μg of rhAS3MT. The assay mixture was incubated at 37 °C for 4 h, and then heated at 95 °C for 5 min to terminate the reaction. H_2O_2 was added to the mixture at the final concentration of 3%, and further incubation was carried out for 1 h. Then, 50-fold diluted catalase was added to eliminate excess H_2O_2 from the mixture. A 50 μL aliquot of the filtrate was applied to GS-320HQ, a multi-mode size exclusion column. The eluate was directly introduced to an ICP–MS to detect Se at *m/z* 82. Se standards, such as iSe^{IV}, iSe^{VI}, and TMSe without treatment with H_2O_2, were also analyzed (C).

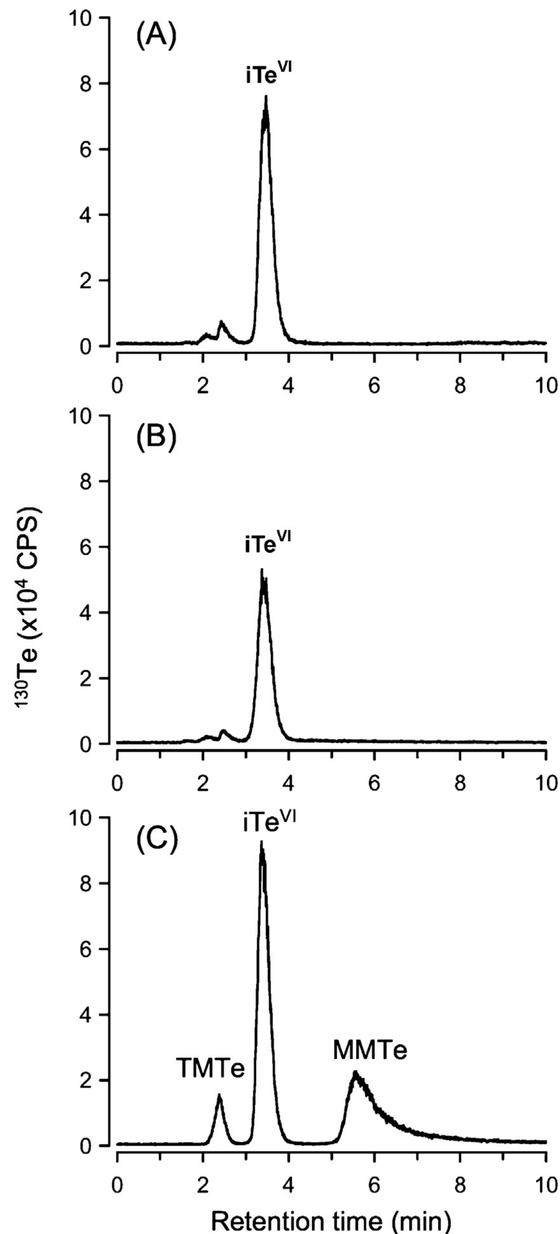

Fig. 3. Elution profile of Te in the assay mixture. The assay mixture consisted of 7 mM GSH, 1 mM SAM as the methyl group donor, and 1 μM iTe^{IV} in 20 mM sodium phosphate buffer in the presence (B) or absence (A) of 30 μg of rhAS3MT. The assay mixture was incubated at 37 °C for 4 h, and then heated at 95 °C for 5 min to terminate the reaction. H_2O_2 was added to the mixture at the final concentration of 3%, and further incubation was carried out for 1 h. Then, 50-fold diluted catalase was added to eliminate excess H_2O_2 from the mixture. A 50 μL aliquot of the filtrate was applied to ES-502 N 7 C, an anion exchange column. The eluate was directly introduced to an ICP–MS to detect Te at *m/z* 130. Te standards, such as iTe^{IV}, MMTe, and TMTe without treatment with H_2O_2, were also analyzed (C).

toxicity. It has been reported that some harmful metabolites, such as monomethylarsonous acid ($MMAs^{III}$) and dimethylarsinous acid ($DMAs^{III}$), are formed by the methylation of iAs^{III} [24]. Those trivalent methylated arsenicals possess comparable toxicity to iAs^{III}. Indeed, the IC_{50} values of iAs^{III} and $DMAs^{III}$ in human epidermoid carcinoma A431 cells were 5.49 and 2.16 μM, respectively [25]. In addition to the trivalent methylated arsenicals, such thioarsenicals as dimethylmonothioarsinic acid ($DMMTAs^{V}$) and dimethyldithioarsinic acid ($DMDTAs^{V}$) are also reported as

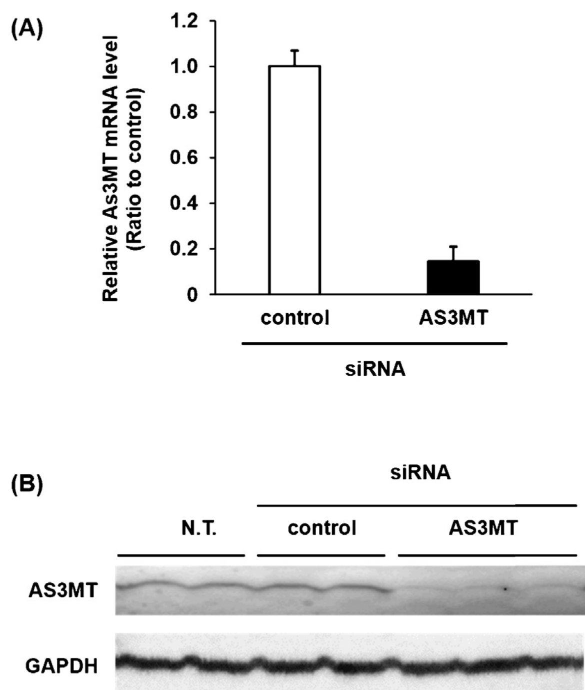

Fig. 4. Effect of siRNA targeting AS3MT on AS3MT mRNA and protein expression. AS3MT mRNA and protein expression was analyzed by real-time RT–PCR and Western blotting, respectively. Columns and bars represent the means ± S.D. of the relative amounts of AS3MT mRNA for three samples (A). The mRNA expression of AS3MT was normalized to that of GAPDH. Two typical blots of AS3MT protein are shown (B). N.T.; not transfected.

harmful metabolites of iAs^{III} even though they are pentavalent species [26]. The IC_{50} values of $DMMTA^{V}$ in human epidermoid carcinoma A431 cells and human bladder carcinoma EJ-1cells were 10.7 and 16.7 μM, respectively [25,27]. Consequently, the toxicity of iAs^{III} in AS3MT-knockdown cells would be comparable to those of harmful metabolites in control cells. However, $DMAs^{V}$ as the final metabolite is far less toxic than those harmful metabolites because its IC_{50} in A431 cells is reported to be 843 μM [13]. Thus, this speculation seems to be less feasible than the next one. As our second speculation, AS3MT is not a specific factor for the methylation of As. AS3MT-KO mice are available and are known to be more sensitive to the As toxicity than wild-type mice [28,29]. Nonetheless, it was reported that the methylated metabolites of As were detected in urine, liver, and kidney of even AS3MT-KO mouse, although the amounts of the metabolites in AS3MT-KO mouse were less than those in wild-type mouse [28]. This observation also suggested that AS3MT was not the sole factor catalyzing the methylation of As, *i.e.*, other enzyme(s) might exist. Indeed, it was reported that N-6 adenine-specific DNA methyltransferase 1 (N6AMT1) involved in the methylation of $MMAs^{III}$ to DMA although its expression was relatively low comparing with AS3MT [30]. Future studies should be conducted to entire mechanism underlying the biomethylation of metalloids in cultured cells.

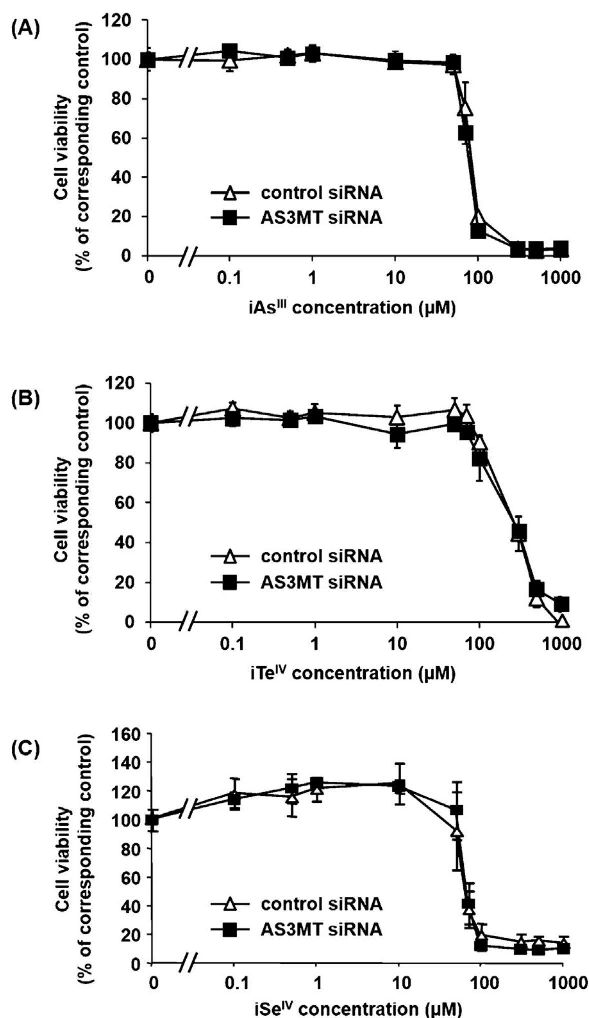

Fig. 5. Effect of siRNA targeting AS3MT on the cytotoxicity of arsenite, selenite, and tellurate. HepG2 cells were transfected with AS3MT-targeting siRNA or control siRNA, and exposed to iAs^{III} (A), iSe^{IV} (B), or iTe^{IV} (C) for 48 h after the transfection at concentrations of 0, 0.5, 1.0, 10, 50, 70, 100, 300, 500, and 1000 μM for 24 h. Values and bars indicate the means ± S.D. for four samples.

4. Conclusions

AS3MT specifically catalyzes the methylation of As, and is not responsible for the methylation of other biomethylatable metalloids, such as Se and Te. Other methylation enzyme(s) may catalyze the methylation of As in HepG2 cells.

Conflict of interest

None.

Transparency document

The Transparency document associated with this article can be found in the online version.

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