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Alternative splicing variants of human arsenic (+3 oxidation state) methyltransferase

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ABSTRACT

Arsenic (+3 oxidation state) methyltransferase (As3MT) catalyzes the methylation of trivalent arsenic (As(III)) to monomethylarsonate (MMA(V)) and dimethylarsinic acid (DMA(V)), and plays an important role in the detoxification of arsenicals. Here, we report the identification of two splicing variants of the human As3MT gene. One splicing variant was an exon-3 skipping (Δ3) form which produced a premature stop codon, and the other was an exon-4 and -5 skipping (Δ4,5) form which produced a 31.1 kDa As3MT protein. In addition to the full-length mRNA of As3MT, Δ4,5 mRNAs were detected in HepG2, A549, HL60, K562, and HEK293 cells. The methyltransferase activity of the recombinant Δ4,5 As3MT and wild-type As3MT proteins purified from *Escherichia coli* was determined. Speciation analysis by HPLC–ICP-MS showed a clear peak of MMA(V) after incubation of As(III) with the wild-type As3MT protein, but not with the Δ4,5 As3MT protein. In addition, COS-7 cells transfected with Δ4,5 As3MT cDNA did not convert As(III) to MMA(V) or DMA(V). The lack of methyltransferase activity of Δ4,5 As3MT seems to be related to the deletion of an *S*-adenosylmethionine-binding site and a critical cysteine residue. These data suggest that the expression pattern of splicing variants of the As3MT gene may affect the capacity for arsenic methylation in cells.

1. Introduction

Arsenic is a metalloid that is ubiquitously distributed in the Earth's crust. High levels of arsenic contamination of groundwater in East Asia, particularly in Bangladesh, Vietnam, and China, have led to chronic arsenic exposure in over 30 million people and caused severe health effects [1]. On the other hand, arsenicals have been used therapeutically in Chinese medicine since ancient times. Over the last decade, arsenic trioxide (As_2O_3) was adopted for the treatment of acute promyelocytic leukemia (APL) patients. Administration of low-dose As_2O_3 resulted in the induction of complete remission in relapsed APL patients [2].

In mammals, arsenite (As(III)) undergoes extensive methylation to yield monomethylarsonous acid (MMA(III)), monomethylarsonate (MMA(V)), dimethylarsinous acid (DMA(III)) and dimethylarsinic acid (DMA(V)) [3–5]. These methylated arsenic metabolites result from the reaction catalyzed by arsenic (+3 oxidation state) methyltransferase (As3MT) [6]. Such metabolic pathways have been considered to be involved in the detoxification process of inorganic arsenicals; however, recent studies have indicated that MMA(III) and DMA(III) are more toxic than inorganic arsenicals [7–9]. Thus, the contribution of As3MT in arsenic detoxification remains controversial. Cohen and his collaborators investigated the roles of As3MT using As3MT knockout mice [10–12]. When exposed to As(III) contained in the diet or drinking water, As3MT knockout mice showed high systemic toxicity in the bladder epithelium, mild acute inflammation in the liver, and hydronephrosis in the kidneys, and the incidences of these tissue damages in As3MT knockout mice were higher than those in WT mice [10,11]. Although these reports indicate that As3MT activity plays an important role in the modulation of arsenic toxicity, little is known about the mechanisms underlying the regulation of As3MT activity and of As3MT gene expression.

Alternative splicing generates more than two mRNAs by alteration in the location and combination at the splicing sites, resulting in variant isoforms of the protein translated from a single gene. Also, a premature stop codon appears infrequently by alternative splicing, and has been implicated in some severe neurological disorders [13,14]. For example, some types of spinal muscular atrophy and Duchenne muscular dystrophy are caused by the exon-7 skipping of the survival motor neuron 2 gene and exon-31 skipping of the dystrophin gene, respectively [15,16]. However, the mechanisms by which alternative splicing is regulated are poorly understood.

Here, we first report the identification of two splicing variants of the human As3MT gene. One of the two splicing variants of

Abbreviations: As(III), inorganic arsenite; DMA(V), dimethylarsinic acid; HPLC–ICP-MS, high performance liquid chromatography–inductively coupled plasma-mass spectrometry; MMA(V), monomethylarsonate; SAM, *S*-adenosylmethionine; WT, wild-type.

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human As3MT does not have As(III)-methyltransferase activity. In addition, we found that the mRNA levels of splicing variants were varied in human-derived cultured cells.

2. Materials and methods

2.1. Materials

Sodium arsenite (As(III)), sodium arsenate and dimethylarsinic acid (DMA(V)) were purchased from Wako Pure Chemicals (Osaka, Japan). Antibody for Xpress was purchased from invitrogen (Carlsbad, CA). Monomethylarsonate (MMA(V)) was purchased from Tri Chemical Laboratories (Yamanashi, Japan). All other reagents and chemicals used were of the highest grade available.

2.2. RT-PCR

The total RNA was isolated with Sepasol-RNA I Super (Nacalai Tesque, Kyoto, JAPAN). The RT-PCR experiments were conducted with a SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (invitrogen). The reaction mixture was incubated at 45 °C for 30 min for reverse transcription. Then, PCR reactions were carried out as following protocol: 1 cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 2 min, and a final cycle at 68 °C for 5 min. The primers sequence were 5′-CAGGATCCCATGGCTGCACTTCGTGACGCTGAGA-3′ and anti-sense primer 5′-GGCTCGAGTTAGCAGCTTTTCTTTGTGCCACAGC-3′ (underlined letters in the sense and anti-sense primers indicate the translation initiation and termination codons, respectively). The amplified products were resolved by 1% agarose gel electrophoresis.

2.3. Cell culture

HepG2, A549, HL60, K562, HEK293, and COS-7 cells were obtained from ATCC (Manassas, VA). HepG2, A549, HEK293 and COS-7 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 using Dulbecco's modified Eagle's medium (Wako Pure Chemicals) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). HL60 and K562 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 using RPMI-1640 (Wako Pure Chemicals) containing 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.4. Cloning of human As3MT

The human As3MT cDNA was amplified with total RNA extracted from HepG2 cells by RT-PCR. The total RNA was isolated, and 3 μg of total RNA was reverse-transcribed to the first strand cDNA for 1 h at 42 °C with PrimeScript™ Reverse Transcriptase (Takara, Shiga, Japan). Amplification of these cDNAs by PCR was performed with PrimeSTAR™ Max DNA Polymerase (Takara) using the same primers as described above. Amplified cDNA was cloned using a StrataClone Blunt PCR cloning Kit (Agilent Technologies, Palo Alto, CA). The sequenced cDNAs of WT As3MT and Δ4,5 As3MT were subcloned into pRSET-B (invitrogen) to obtain His-Xpress-tagged protein in *Escherichia coli*, and into pcDNA3.1(+) or pcDNA3.1/HisB (invitrogen) for overexpression of the human As3MT protein in mammalian cells.

2.5. Expression and purification of human As3MT

The cloned WT/pRSET-B and Δ4,5/pRSET-B plasmids were transformed into BL21(DE3)pLysS (Promega, Madison, MI). Selected transformed cells were grown in 10 mL SOB medium (20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.186 g KCl, and 10 mM $MgSO_4$ per 1 L) containing 100 μg/mL of ampicillin and 35 μg/mL of chloramphenicol for 16 h at 37 °C. The cultured cells were transferred into 100 mL of fresh SOB medium and grown at 37 °C until the optical density at 600 nm reached 0.3–0.4. The WT As3MT protein was induced by incubation with 1 mM IPTG for 8 h at 37 °C. For preparation of the recombinant Δ4,5 As3MT protein, the incubation was carried out with 10 mM IPTG for 2 days at 15 °C. The cultured cells were harvested by centrifugation at 8000 rpm for 10 min at 4 °C, and then resuspended in binding buffer (20 mM Tris–HCl (pH 7.9), 500 mM NaCl containing 20 mM imidazole). After sonication on ice and centrifugation at 13,000 rpm for 10 min at 4 °C, the lysates were loaded onto an Ni Sepharose 6 Fast Flow column (GE Healthcare, Buckinghamshire, UK) which had been equilibrated with the binding buffer to purify the target protein. The recombinant protein was eluted with elution buffer (20 mM Tris–HCl (pH 7.9), 500 mM NaCl containing 500 mM imidazole). The purity of each protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting and Coomassie brilliant blue (CBB) staining. Each protein was dialyzed twice against 20 mL potassium phosphate buffer (pH 7.0) to remove imidazole. The protein concentration was determined by a BCA protein assay kit (Thermo Scientific, Rockford, IL).

2.6. Western blot

The recombinant protein purified from *E. coli*, and total lysates extracted from COS-7 cells were used for Western blotting with As3MT, which produced by our laboratory, and Xpress antibodies. Western blotting was performed as described previously [17].

2.7. Assay of As3MT activity

The As3MT enzyme activity was determined by using HPLC–ICP-MS. Reactions were carried out in 100 μL of reaction mixture containing 20 mM potassium phosphate buffer (pH 7.0), 30 μg recombinant protein, 100 mM SAM, 7 μM GSH, and 10 μM As(III). The reaction mixtures were incubated at 37 °C for 6 h followed by 95 °C for 5 min. The samples were then treated with hydrogen peroxide at a final concentration of 3% at 37 °C for 1 h followed by 95 °C for 5 min. After the filtration of the sample with an Amicon YM-3 (Millipore, Bedford, MA) at 14,000*g* for 30 min, 5 μL of filtrates was separated by a NANOSPACE HPLC system (Shiseido, Tokyo, Japan) on a CAPCELL PAK C18 MGII (1.0 mm i.d. × 150 mm long) (Shiseido) using 5 mM tetrabutylammonium hydroxide, 3 mM malonic acid, and 4% methanol, as a mobile phase with a flow rate of 200 μL/min. The eluates from the HPLC column were directly introduced into the ICP-MS spray chamber via a PEEK tube (0.13 mm i.d.) (HP4500 plus; Yokokawa Analytical Systems, Tokyo, Japan). Arsenic in the eluates was monitored at *m/z* 75. The peaks of As(V), MMA(V), and DMA(V) were identified by comparison of the retention times for each standard arsenic compound: sodium arsenate, monomethylarsonate and dimethylarsinic acid, respectively.

2.8. Transient transfection

COS-7 cells were seeded at 4×10^5 cells per well in 6-well plates and transfected with 1.5 μg/well of each plasmids, together with 10 μL/well Polyfect transfection reagent (QIAGEN, Valencia, CA). To examine the As3MT protein expression by Western blotting, cell lysates were extracted 24 h after the transfection of cDNAs (WT/pcDNA3.1(+), WT/pcDNA3.1/His, Δ4,5/pcDNA3.1(+), and Δ4,5/pcDNA3.1/His). The transfected COS-7 cells were incubated for 24 h, and then exposed to 20 μM As(III) for 24 h. The cell lysates suspended with 100 μL of 150 mM Tris–HNO_3 were then sonicated and incubated at 70 °C for 30 min to inactivate cellular

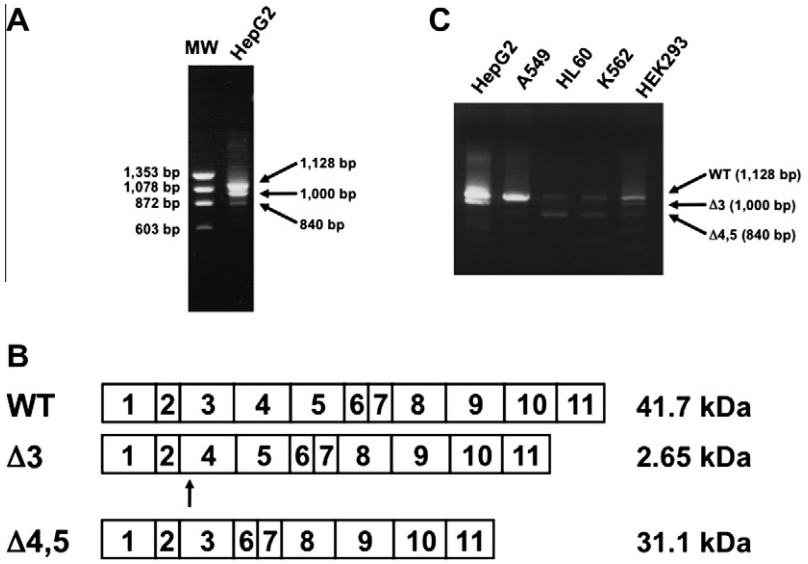

Fig. 1. Splicing variants of human As3MT mRNA. (A) RT-PCR was performed in HepG2 cells. MW shows a ΦX174 DNA-HaeIII digest molecular weight marker. (B) The diagram of the full-length, Δ3, and Δ4,5 splicing variants of As3MT mRNA. The anticipated molecular weights of the translated proteins from the Δ3 and Δ4,5 As3MT mRNAs are 2.65 kDa and 31.1 kDa, respectively. The arrow indicates the site of premature stop codon in Δ3 As3MT mRNA. (C) mRNA expression of As3MT splicing variants were examined by RT-PCR in human-derived cultured cells.

catalase, and hydrogen peroxide was added to a final concentration of 10% at room temperature for 3 h [18]. Each of these samples was centrifuged at 15,000*g* for 10 min at 4 °C, and the supernatant was applied to an Amicon YM-3 centrifugal filter at 15,000*g* for 20 min at 4 °C. The eluates were analyzed for arsenic speciation by HPLC–ICP-MS as described above.

3. Results

When we carried out RT-PCR with total RNA extracted from HepG2 cells and the primers containing the initiation and termination codons of human As3MT, we found that three PCR products were amplified (Fig. 1A), suggestive of alternative splicing. Therefore, we determined DNA sequences of the three PCR products following the ligation of each PCR product to the sequencing vector. Results obtained from DNA sequence revealed that 1128, 1000, and 840 bp cDNAs represent a full-length (WT) As3MT mRNA, a splicing variant in which exon-3 is deleted (Δ3 As3MT), and a splicing variant in which both exon-4 and -5 are deleted (Δ4,5 As3MT), respectively (Fig. 1B). The WT As3MT protein was calculated to be 41.7 kDa (375 amino acids), while the Δ4,5 As3MT protein was 31.1 kDa (279 amino acids). On the other hand, the Δ3 As3MT protein was calculated to be 2.65 kDa (23 amino acids) due to the appearance of a premature stop codon derived by frame shift (Fig. 1B, arrow). To our knowledge, this is the first observation of splicing variants in products of the human As3MT gene.

To examine whether As3MT splicing variants are also expressed in other human-derived cell lines than HepG2 cells, RT-PCR analysis was performed with lung carcinoma A549 cells, leukemia HL60 and K562 cells, and embryonic kidney HEK293 cells. Interestingly, the PCR products indicative of Δ4,5 As3MT mRNA were detected in all cell lines examined, although the abundance of WT and Δ4,5 mRNAs was varied among the cell lines (Fig. 1C).

In order to investigate whether the product of Δ4,5 As3MT mRNA possesses As(III)-methyltransferase activity, we prepared recombinant WT and Δ4,5 As3MT proteins in *E. coli*. The purification of the recombinant WT and Δ4,5 As3MT protein was confirmed by Western blotting and CBB staining (Fig. 2A), and the As(III)-methyltransferase activity of these purified proteins was examined in a cell-free system. Arsenic speciation with HPLC-ICP-MS showed a clear peak of MMA(V) after the incubation of As(III) with the recombinant WT As3MT protein (Fig. 2B). However, the peaks of methylated arsenic species were not detected after the incubation with the recombinant Δ4,5 As3MT protein. These results indicate that the Δ4,5 As3MT protein lacks As(III)-methyltransferase activity.

To further examine the enzyme activity of the As3MT splicing variants, we transfected WT or Δ4,5 As3MT cDNA into COS-7 cells and examined the As(III)-methyltransferase activity in COS-7 cells. We first confirmed the transient expression of WT and Δ4,5 As3MT proteins after the transfection with each plasmid into COS-7 cells. As shown in Fig. 3A, the WT and Δ4,5 As3MT proteins were detected 24 h after the transfection regardless of the presence or absence of the His-Xpress tag. Exposure of WT As3MT cDNA-transfected COS-7 cells to As(III) for 24 h resulted in an obvious peak of DMA(V). On the other hand, no peaks of methylated arsenic species were detected in Δ4,5 As3MT cDNA-transfected COS-7 cells exposed to As(III) (Fig. 3B). Taken together, these data indicate that the Δ4,5 As3MT protein lacks the enzyme activity to convert As(III) into methylated arsenic species both in a cell-free system and in the transfected cells.

4. Discussion

This is the first report showing that human As3MT has two splicing variants. A premature stop codon appears in the Δ3 splicing variant, and the Δ4,5 splicing variant is translated until an intrinsic stop codon, but lacks the As(III)-methyltransferase activity. In addition, we detected the mRNAs of both WT and Δ4,5 As3MT in several human-derived cultured cells.

In this study, we found that Δ4,5 As3MT does not have As(III)-methyltransferase activity. Hamahata et al. [19] showed that site-directed mutagenesis in the conserved domain (Motif I) of rat guanidinoacetate methyltransferase resulted in a loss of enzyme activity due to the inability to bind SAM. The glycine-rich sequence of Motif I is reminiscent of the nucleotide-binding motifs in dehydrogenases and kinases [20]. The Motif I of human As3MT is located within the amino acid sequence translated from exon-4 and -5 of

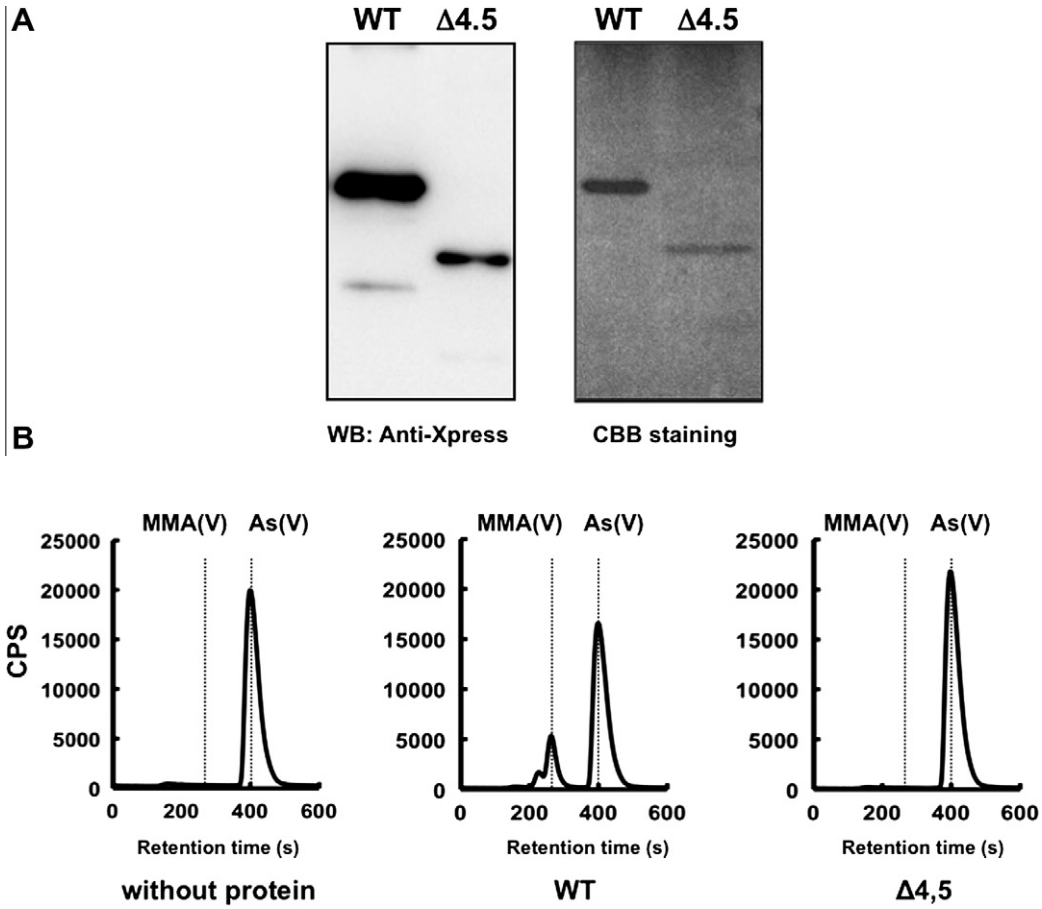

Fig. 2. As3MT activity in the recombinant WT and Δ4,5 protein. (A) Western blot with anti-Xpress antibody (left) and CBB staining (right) of the recombinant WT and Δ4,5 As3MT proteins. (B) Arsenic speciation in samples reacted with recombinant WT or Δ4,5 As3MT protein, SAM, GSH and As(III).

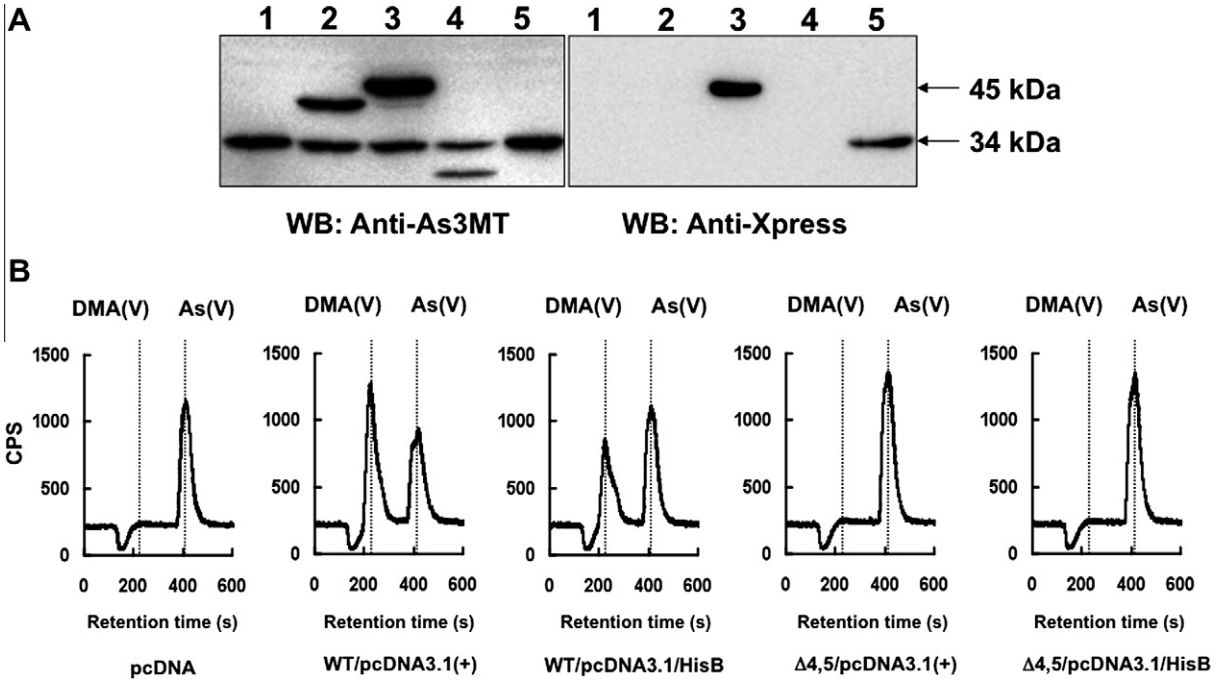

Fig. 3. Arsenic speciation in As3MT cDNA-transfected COS-7 cells. (A) The lysates from COS-7 cells transfected with WT/pcDNA3.1(+), WT/pcDNA3.1/HisB, Δ4,5/pcDNA3.1(+), or Δ4,5/pcDNA3.1/HisB cDNA for 24 h were applied to a Western blot with anti-As3MT (left) or anti-Xpress (right) antibody. (B) Arsenic speciation in cell lysates after exposure of transfected COS-7 cells to 20 μM As(III) for 24 h.

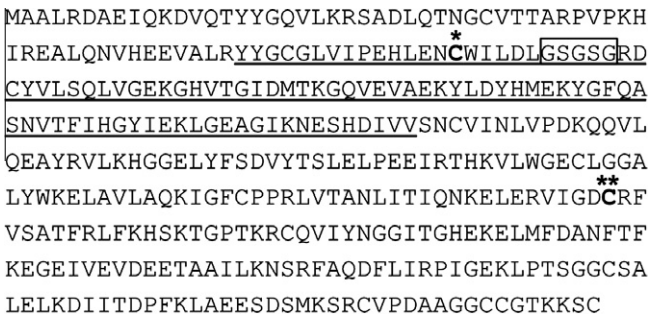

Fig. 4. Human As3MT amino acid sequence. C*, Cys72; C**, Cys250. A boxed region indicates a glycine-rich SAM-binding motif. Underlined sequences of amino acids are coded by exon-4 and -5 in the mRNA.

the mRNA (Fig. 4). Therefore, it seems likely that the Δ4,5 As3MT protein does not have methyltransferase activity due to the deletion of the SAM-binding domain. In addition, Song et al. [21] demonstrated that replacement of Cys72 with serine by site-directed mutagenesis resulted in a complete loss of As3MT activity, and suggested that Cys72 may form a critical intramolecular disulfide bond with Cys250, which is considered to play an important role in the enzymatic function and structure of As3MT [22]. Since the codon for Cys72 is located within exon-4 of the mRNA, Δ4,5 As3MT might have become inactive because of a double deletion of the Cys72 for disulfide bond formation and the glycine-rich motif for SAM-binding (Fig. 4).

Alternative splicing is regulated by *cis*-acting elements such as the exonic and intronic enhancer or silencer embedded in premature mRNA, and by *trans*-acting elements such as RNA-binding proteins that form a spliceosome. Recent advances in genome-wide sequencing of human genes have demonstrated that alternative splicing is more deeply involved in a variety of biological events than previously expected [23,24]. Importantly, the deregulations in alternative splicing result in aberrantly spliced variants that contribute to development of neurological disorders [25], muscular diseases [26], and cancer [27,28]. Thus, it seems likely that the occurrence of splicing variants of As3MT in the cancerous cell lines used in this study was caused by aberrant splicing of the premature As3MT mRNA during the process of carcinogenesis. In this regard, it is noteworthy that Δ4,5 As3MT, which lacks As(III)-methyltransferase activity, is expressed in human leukemia HL60 and K562 cells since As_2O_3 is adopted for the treatment of APL [2]. Further studies are required to clarify the protein expression of each splicing variants of As3MT among human leukemia cells and its relevance to enzymatic methylation of As_2O_3.

Several studies have reported the association of genetic polymorphisms in the human As3MT gene with the activity and expression of As3MT and its relevance to the impact on arsenic metabolism among the people living in arsenic-contaminated areas [29–32]. For example, it has been reported that an exonic variant (Met287Thr) [33,34] and noncoding variants significantly affect arsenic metabolism [32,35,36]. In addition, Engstrom et al. [30] reported that noncoding As3MT gene polymorphisms influence As3MT gene expression in peripheral blood cells. However, little is known about the splicing variants of the As3MT gene, and no study has investigated the relationship of As3MT splicing variants with arsenic metabolism in a population living in an arsenic-polluted area. Further studies are required to assess the frequency of alternative splicing of the As3MT gene among different populations and its role in individual differences in the capacity for arsenic methylation.

Taken together, we consider it important to monitor how the expression patterns of alternative splicing variants of the As3MT gene vary among different tissues, individuals and durations of exposure to arsenic, as well as to monitor the development of malignant alterations.

Conflict of interest statement

None declared.

Acknowledgments

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