Role of arsenic disulfide in the demethylation of *PTPL1* in diffuse large B cell lymphoma cells (#84081)

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Role of arsenic disulfide in the demethylation of *PTPL1* in diffuse large B cell lymphoma cells

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Background. The expression and methylation status of *PTPL1* gene in diffuse large B cell lymphoma (DLBCL) cells is not well defined, and the effect of arsenic disulfide on *PTPL1* methylation is still unclear.

Methods. Based on two DLBCL cell lines (i.e. DB and SU-DHL-4 cells), we knocked down the expression of *PTPL1* using siRNA, and then investigated the role of *PTPL1* in DLBCL progression. The methylation status of *PTPL1* in DLBCL cells was analyzed by MSPCR. We then analyzed the effects of different doses of arsenic disulfide on *PTPL1* methylation.

Results. The results showed that *PTPL1* knockdown promoted the proliferation of DLBCL cells. *PTPL1* was hypermethylated in DLBCL cells. Arsenic disulfide could reverse *PTPL1* methylation in a dose-dependent manner, which may be related to the inhibition of DNA methyltransferases (DNMTs) and the increase of methyl-CpG-binding domain 2 (MBD2).

Conclusion. *PTPL1* may a tumor suppressor gene in DLBCL progression. *PTPL1* methylation could be reversed by arsenic disulfide in a dose-dependent manner. Our study may provide a theoretical basis for the clinical application of arsenic disulfide in DNA methylation-related diseases.

Role of arsenic disulfide in the demethylation of *PTPL1* in diffuse large B cell lymphoma cells

Short Title: *PTPL1* demethylation by As_2S_2 in DLBCL

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Abstract

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Conclusion. *PTPL1* may be a tumor suppressor gene in DLBCL progression. *PTPL1* methylation could be reversed by arsenic disulfide in a dose-dependent manner. Our study may provide a theoretical basis for the clinical application of arsenic disulfide in DNA methylation-related diseases.

Introduction

Diffuse large B cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin's lymphoma in adults accounting for about 30-40% (*Goldfinger and Cooper, 2022*). It can be induced by various factors, including gene mutation, chromosomal translocation, and rearrangement, as well as cell cycle dysregulation, excessive cell proliferation and apoptosis inhibition induced by abnormal extragenic regulatory systems and signal transduction pathways (*Schmitz et al., 2018*). However, the pathogenesis of DLBCL has not been well elucidated. The clinical manifestations of DLBCL patients are diverse with a high heterogeneity in morphology, genetics, immunophenotype, and prognosis, which results in different responses to treatment. Specifically, about 20-40% of patients show no response or even rapid progression and relapse after treatment (*Chapuy et al., 2018*). Therefore, the current priority on DLBCL is to find effective therapeutic targets.

Gene methylation is closely related to gene expression regulation, cellular proliferation, differentiation, and apoptosis. Studies have found that DNA hypermethylation in tumor suppressor genes could lead to transcriptional silencing, thereby leading to the loss or attenuation of checkpoint functions (*Lopez et al., 2022*).. Recently, promoter hypermethylation has been frequently reported in tumor suppressor genes such as *P16*, *P15*, *P57*, *DAPK*, *PTPL1* and *GSTP1*, and is closely related to the pathogenesis in many malignancies (e.g. human lymphoma) (*Wang et al., 2016*). For example, promoter methylation of *PTPN13/PTPL1* has been confirmed in a variety of malignant tumors including non-small cell lung cancer, ovarian cancer, prostate cancer, and breast cancer (*Bompard et al., 2002; Castilla et al., 2012; Wang et al., 2022; Wang et al., 2018*).

In DLBCL and follicular lymphoma, hypermethylation could be detected in the majority of the *PTPL1* gene promoter, along with attenuation or silencing of *PTPL1*. In contrast, promoter methylation was rarely detected in lymph nodes or samples of reactive lymphoid hyperplasia (RLH) in healthy individuals (*Wang et al., 2016*).

Demethylation agents-induced activation of tumor suppressor genes has been considered a promising candidate for blocking tumor growth and progression. For instance, arsenic trioxide (ATO, As_2O_3), which induces DNA demethylation, has been utilized as an anti-cancer agent by suppressing cancers of the liver, prostate, and breast apparently through demethylation and apoptosis (*Thomas et al., 2010; Xia et al., 2012*). Arsenic disulfide (As_2S_2), the main component of traditional Chinese medicine (TCM) realgar, has been reported to exhibit similar antitumor effects to ATO with lower toxicity (*Zhao et al., 2018*). However, its role of demethylation in DLBCL remains unclear. In present study, we knocked down *PTPL1* in two DLBCL cell lines (i.e. DB and SU-DHL-4 cells) using siRNA to investigate the role of *PTPL1* in DLBCL progression. Promoter methylation of *PTPL1* in DLBCL cell lines was detected using methylation specific polymerase chain reaction (MSPCR). We then analyzed the demethylation effects of different doses of arsenic disulfide on *PTPL1* methylation. This may improve the understanding of the anti-tumor mechanism of arsenic disulfide for the treatment of DLBCL.

Materials & Methods

Cell culture

Two DLBCL cell lines (i.e. DB and SU-DHL-4 cell lines, generously donated by Qilu Hospital of Shandong University) were cultured in IMDM or RPMI1640 medium containing 10% fetal bovine

serum (FBS) in an incubator with 5% CO_2 at 37°C. Then cells in the logarithmic growth phase were collected for the subsequent analysis.

Reverse transcription PCR (RT-PCR)

Total RNA was extracted from DB and SU-DHL-4 cells using TRIzol reagent (ThermoFisher, CA, USA) according to the manufacturer's instructions. After the synthesis of cDNA, reverse transcription amplification of *PTPL1* was conducted with the specific primers (5'-CAACAATGGTCAGCAACAG-3'; 5'-CACCACAAAGCCCTTCA-3'). The amplification conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. GAPDH was used as an internal reference. RT-PCR products were subjected to DNA agarose gel electrophoresis (1.5%) and then were observed using a gel imaging system.

Silencing of PTPL1

To investigate the roles of *PTPL1* in DLBCL, DB and SU-DHL-4 cells were divided into the following groups: i) control group, with no transfection; ii) negative control (NC) group, transfected with randomized control siRNA; iii) siRNA group, transfected with siRNA (sequence, CCACCATGCTGCAATTGAA). Transfection was performed using lipofectamine 2000 (ThermoFisher, CA, USA), according to the manufacturer's instructions. The silencing of *PTPL1* was verified based on RT-PCR and Western blot analysis, respectively.

CCK-8 analysis for cellular proliferation

Cells (1×105/mL, 100μL) in each group were added into the wells of plate and incubated in an incubator with 5% CO_2 at 37℃ for 48h. Afterwards, cells were incubated with 10 μL/well CCK-8 reagents for about 1 h. Absorbance at 450 nm was measured with a microplate reader. The cell

proliferation rate was calculated as the ratio of the OD value in treatment group and the control group.

DNA extraction and methylation

Cells in the logarithmic growth phase were washed twice with PBS, followed by genomic DNA extraction using a commercial kit (Omega, USA) accordance to the manufacturer's instructions. Afterwards, DNA methylation was performed using 200 ng genomic DNA with a commercial kit (Epigentek, USA).

MSPCR

MSPCR was conducted to measure the *PTPL1* methylation as previously described (*Wang et al., 2016*). Briefly, two pairs of primers (primer M: 5'-TATAGAAATAAGGTTGAGAGGTAGC-3', 5'-CGAACGACAAAATTCCTAACG-3'; primer U: 5'-AATATAGAAATAAGGTTGAGAGGTAGT-3'; 5'-ACCAAACAACAAAATTCCTAACAC-3') were used to amplify methylated DNA and non-methylated DNA, respectively. The amplification conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s (for methylated DNA) or 60°C for 30 s (for non-methylated DNA), and 72°C for 30 s, and72°C for 10 min. Finally, the amplified PCR products were subjected to DNA agarose gel electrophoresis (1.5%), followed by observation using a gel imaging system.

PTPL1 gene methylation after AS_2S_2

To investigate the effects of AS_2S_2 on PTPL1 gene methylation, the cell lines were treated with different AS_2S_2 (5μL, 10μL, and 20μL) for 72h, respectively. RT-PCR was used to detect the mRNA levels of DNMTs (i.e. DNMTl and DNMT3) and methyl-CpG-binding domain 2 (MBD2).

115 DNMTl primers were 5’-CAACGGGCAGATGTTTCA-3’ and 5’-
116 TCCTCACATTCATCCACCA-3’. DNMT3B primers were 5’-GAGAAAGCTAGGGTGCGA-3’
117 and 5’-CACTGGTTGCGTGTTGTT-3’. MBD2 primers were 5’-
118 AGTAAGCCCCAGTTGACACG-3’ and 5’-AACTGACACAGGCTGCTTGA-3’. GAPDH (5’-
119 ACAACTTTGGTATCGTGGAAGG-3’ and 5’-GCCATCACGCCACAGTTTC-3’) was used as
120 an internal reference.

121 ***Statistical analysis***

122 SPSS 21.0 was used to statistical analysis. One-way analysis of variance (ANOVA) was used for
123 significance test. The significance level was set at $P < 0.05$.

124 ## Results

125 ***PTPL1 knockdown promoted DLBCL cell proliferation***

126 CCK-8 results showed that PTPL1 knockdown promoted the proliferation of DB and SU-DHL-4
127 cells (**Fig. 1**). This indicated that PTPL1 exhibited the role of suppressing DLBCL.

128 ***PTPL1 methylation in DLBCL cell lines***

129 Methylation was characterized by the appearance of amplification products of primer M.
130 Unmethylation was characterized by the appearance of amplification products of primer U. The
131 amplification products of both primer M and primer U indicated partial methylation. The
132 amplification of primer M was observed in DB and SU-DHL-4 cell lines (**Fig. 2**), indicating the
133 promoter methylation of *PTPL1*.

134 ***Demethylation role of arsenic disulfide on methylated PTPL1***

135 Compared with NC group, the *PTPL1* methylation was attenuated in arsenic disulfide treatment

groups (**Fig. 3**). This indicated that arsenic disulfide exhibited demethylation role, with a dose-dependent manner.

Effects of arsenic disulfide on DNMTl, DNMT3b and MBD2 mRNA expression

RT-PCR results showed that arsenic disulfide significantly decreased the mRNA expression of DNMT1 and DNMT3b and significantly increased the mRNA expression of MBD2 (**Fig. 4**). The effect was proportional to the dose.

Discussion

Studies have confirmed that the dysfunction of tumor suppressor genes promoted the pathogenesis and progression of tumors, resulting in a series of malignant biological characteristics of tumor cells, including self-sufficiency of growth signals, insensitivity to inhibitory growth signals, ability to replicate infinitely, evasion of apoptosis, sustained angiogenesis, promotion of tissue invasion and distant metastasis (*Dromard et al., 2007*). *PTPL1* is located on human chromosome 4q21 and encodes a non-receptor tyrosine phosphatase. Its dephosphorylation can dephosphorylate the tyrosine of tyrosine kinase, thereby antagonizing the growth-promoting effect of tyrosine kinase (*Freiss and Chalbos, 2011*). Numerous studies have shown the abnormal expression of *PTPL1* in various malignant tumors. However, whether *PTPL1* is a tumor suppressor or a tumor promoter is still controversial. *PTPL1* acted as a tumor suppressor in breast cancer and non-small cell lung cancer (*Wang et al., 2022*). In contrast, PTPL1 could inhibit CD95-mediated apoptosis of pancreatic cancer cells and induce drug resistance in head and neck tumors (*Abaan and Toretsky, 2008*). To clarify the role of *PTPL1* in DLBCL, we knocked down *PTPL1* in DB and SU-DHL-4 cell lines using siRNA. The results showed that *PTPL1* knockdown promoted DLBCL cell

proliferation, indicating the inhibitory effect of *PTPL1* on DLBCL.

Hypermethylation of CpG islands located in the DNA promoter has been reported to inhibit the expression of tumor suppressor genes in various tumor cells. Interestingly, unlike genetic alterations, DNA methylation, an epigenetic modification, is reversible (*Kedhari Sundaram et al., 2019*). Therefore, demethylation may restore the expression of tumor suppressor genes, and then inhibit tumor progression, which provides a new idea for the treatment of tumors. Recently, decitabine and azacitidine have been approved for the treatment of hematological malignancies as epigenetic targeting drugs (*Blecua, Martinez-Verbo, & Esteller, 2020*). Nevertheless, numerous patients do not respond to these drugs and eventually relapse (*Bazinet and Bravo, 2022*). Therefore, there is still a need to develop new drugs targeting DNA methylation.

In this study, we detected the *PTPL1* promoter methylation in two DLBCL cell lines (i.e. DB and SUDHL4 cells) using MSPCR method. The data suggested that *PTPL1* was methylated in both cell lines, which may inhibit *PTPL1* expression and promote DLBCL progression. Overexpression of DNA methyltransferases (DNMTs), such as DNMT1, DNMT3A and DNMT3B, could promote DNA hypermethylation, was closely related to the prognosis in cancer patients (*Weisenberger, Lakshminarasimhan, & Liang, 2022*). Besides, MBD2 is a component of the MeCP1 complex and functions as a demethylase (*Feng and Zhang, 2001*). As important TCM components, arsenic drugs, including arsenic disulfide (As_2S_2), arsenic tetrasulfide (As_4S_4), ATO (As_2O_3), exhibit favorable anti-tumor effects in various tumors especially blood-related malignancies (*Wang et al., 2013*). The FDA approved ATO for the treatment of acute promyelocytic leukemia in 2000 (*Jing et al., 1999*). Compared with ATO, arsenic disulfide showed

comparable anti-tumor effects and more advantages, including lower toxicity of oral administration (*Zhao et al., 2019*). To date, the mechanism of arsenic disulfide against tumors is still unclear. In a previous study, arsenic disulfide exerted anti-tumor role by induction of autophagy and apoptosis, as well as cell cycle arrest (*Zhao et al., 2018*). In this study, there was decrease of *PTPL1* gene methylation in cell lines treated with As_2S_2, in a dose dependent manner. For the expression of DNMTs, the RNA expression of DNMT1 and DNMT3b showed significant decrease after As_2S_2 treatment, while the mRNA expression of MBD2 showed significant increase in these cells. These suggested that the inhibition of DNMTs and the increase of MBD2 were potential mechanisms of arsenic disulfide-induced PTPL1 demethylation.

This study has some limitations. First, the optimal dose for arsenic disulfide demethylation remains unclear. Secondly, although we confirmed the demethylation role of arsenic disulfide on methylated *PTPL1*, the exact mechanisms are still not well defined. In the future, more studies are required to illustrate them.

Conclusion

In summary, *PTPL1* was a tumor suppressor gene in DLBCL progression. *PTPL1* methylation could be reversed by arsenic disulfide in a dose-dependent manner. Our data may provide a reference for the clinical application of arsenic disulfide in DNA methylation-related diseases and provide ideas for DLBCL treatment.

Acknowledgement

None.

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283

284 **Figure Legends**

285 **Figure 1.** Cell proliferation and proliferation rate of control group, NC group and siRNA2 group

286 in DB and SU-DHL-4 cell lines. *$P < 0.05$ and **$P < 0.01$.

287 **Figure 2.** *PTPL1* methylation in DB and SU-DHL-4 cell lines detected by MSPCR.

288 **Figure 3.** *PTPL1* methylation in DB and SU-DHL-4 cells treated with different doses of arsenic

289 disulfide detected by MSPCR.

290 **Figure 4.** DNMTl, DNMT3b, and MBD2 mRNA expression in DB and SU-DHL-4 cells treated

291 with different doses of arsenic disulfide detected by RT-PCR. *$P < 0.05$ and **$P < 0.01$.

Figure 1

Figure 1.

Cell proliferation and proliferation rate of control group, NC group and siRNA2 group in DB and SU-DHL-4 cell lines. *$P < 0.05$ and **$P < 0.01$.

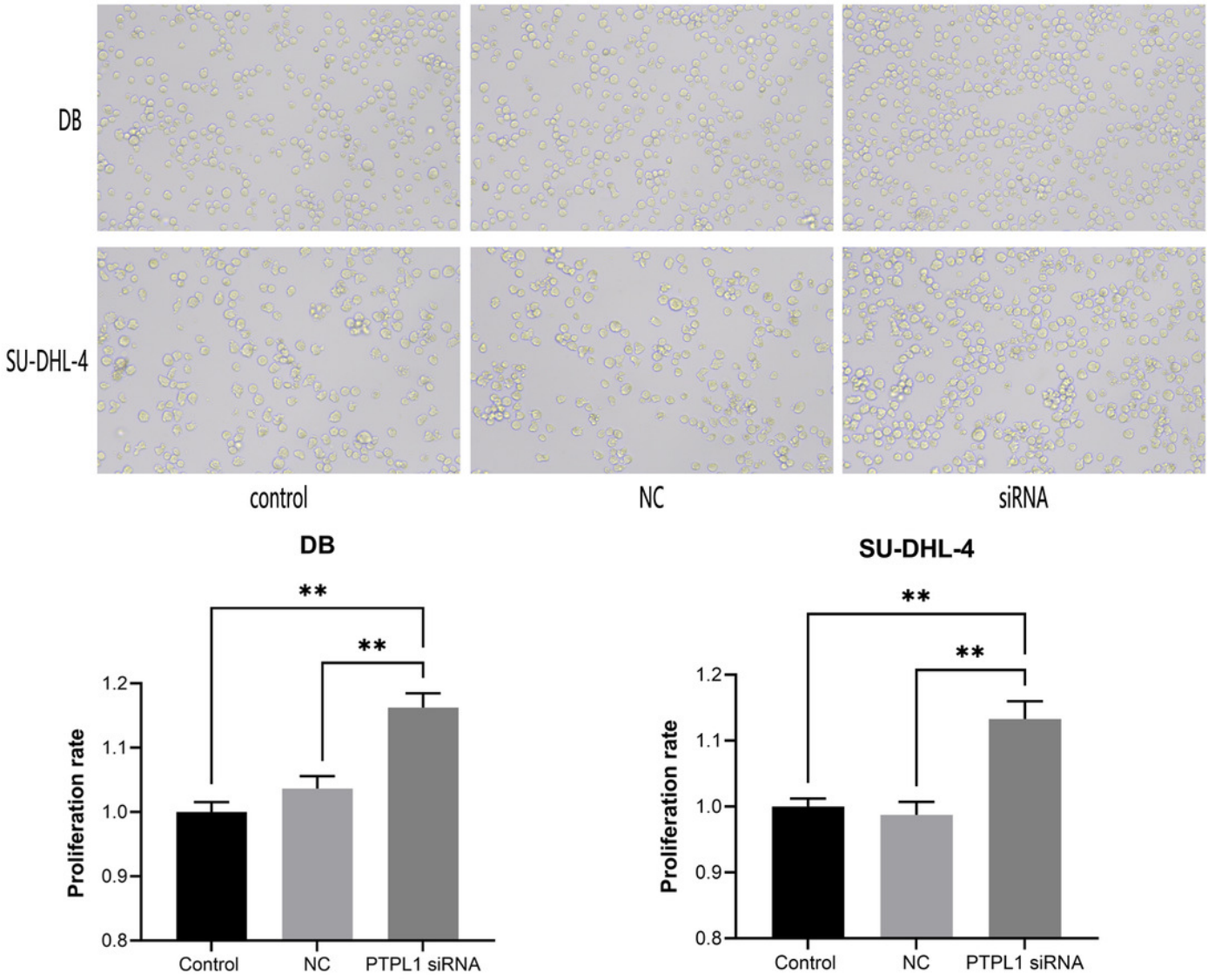

Figure 2

Figure 2.

PTPL1 methylation in DB and SU-DHL-4 cell lines detected by MSPCR.

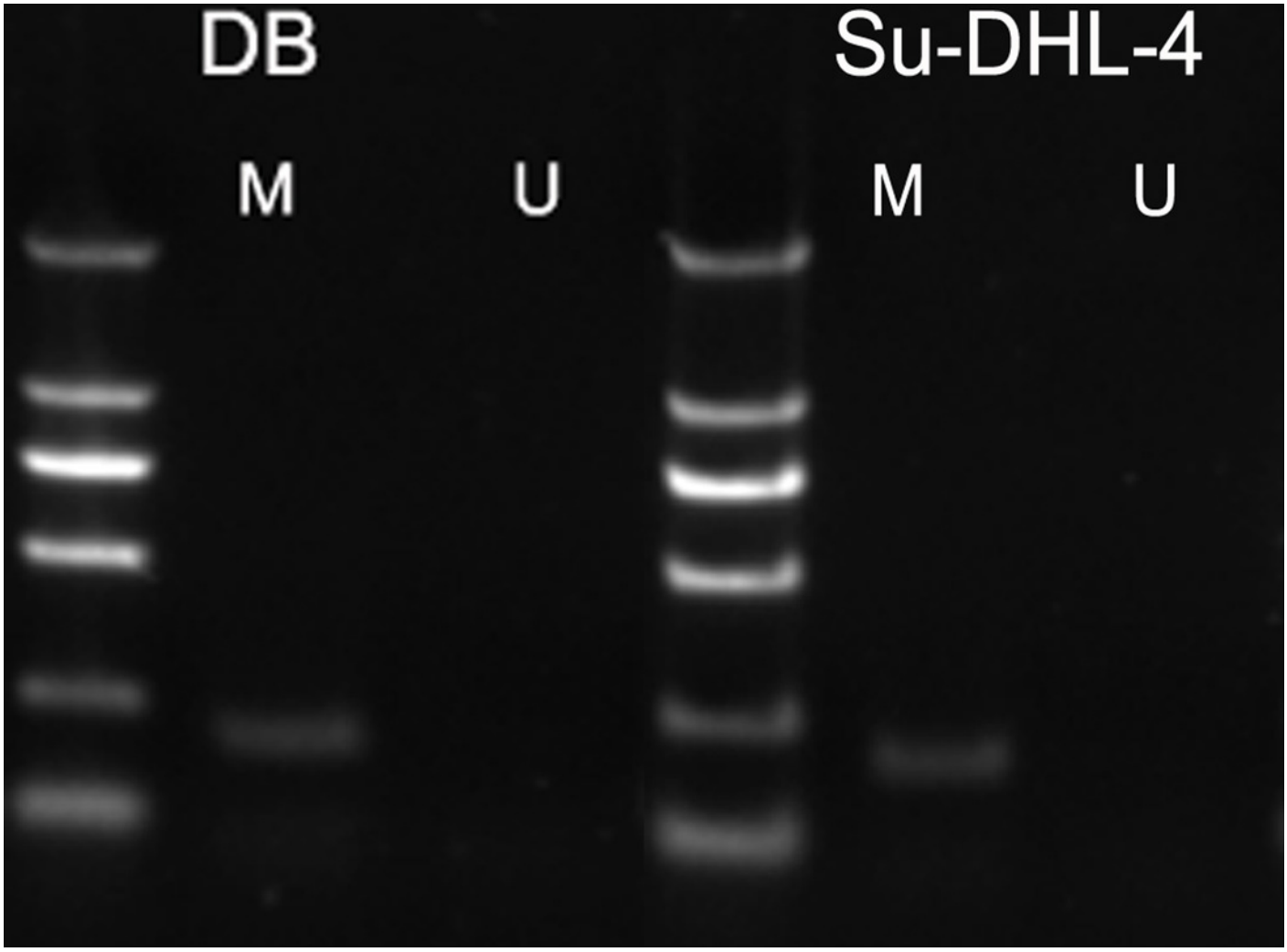

Figure 3

Figure 3.

PTPL1 methylation in DB and SU-DHL-4 cells treated with different doses of arsenic disulfide detected by MSPCR

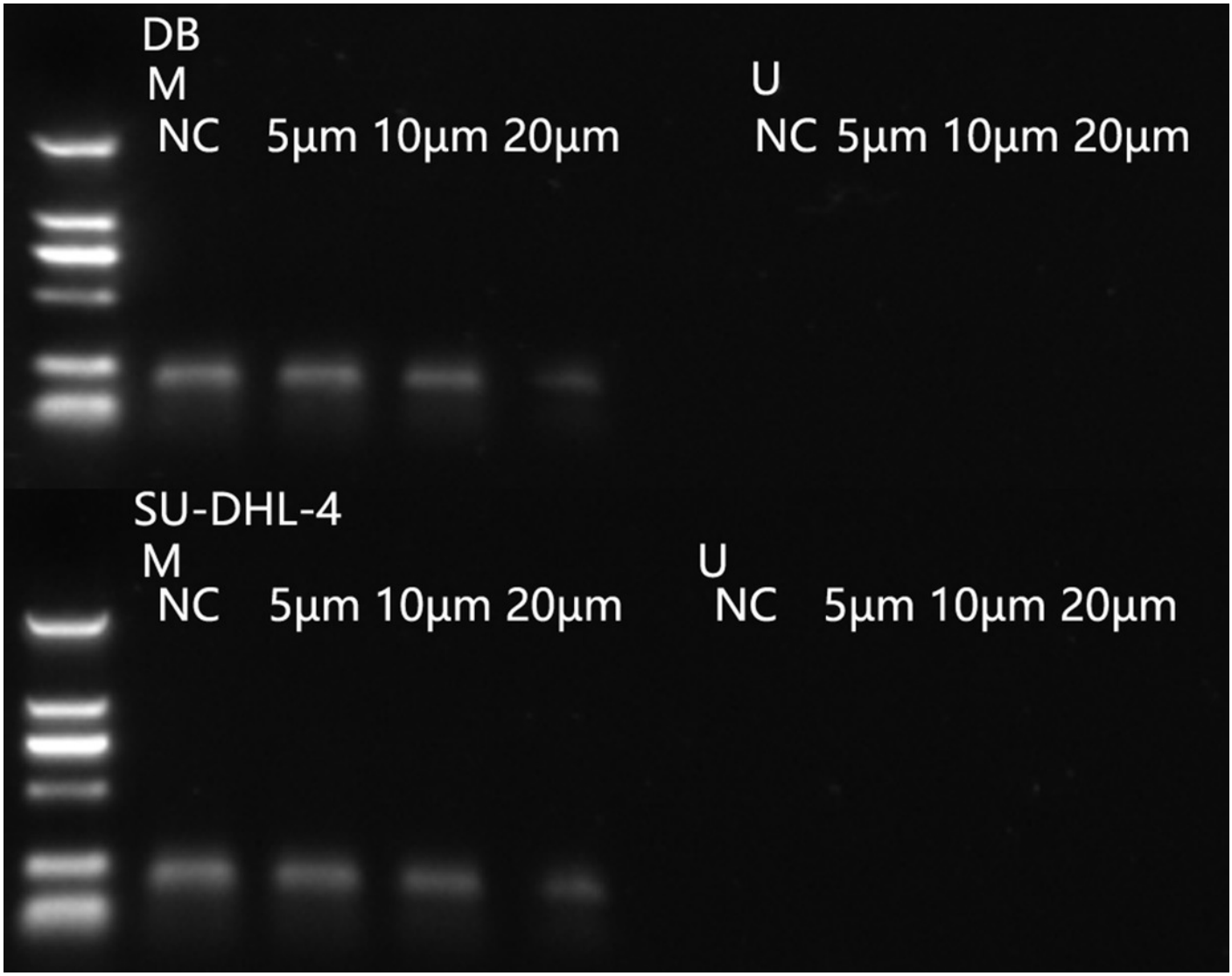

Figure 4

Figure 4.

DNMTl, DNMT3b, and MBD2 mRNA expression in DB and SU-DHL-4 cells treated with different doses of arsenic disulfide detected by RT-PCR. *$P < 0.05$ and **$P < 0.01$.