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Loss of an ABC transporter in *Arabidopsis thaliana* confers hypersensitivity to the anti-cancer drug bleomycin

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ABSTRACT

Bleomycin (BLM) is used as an anti-cancer drug clinically. However, some cancer cells are resistant to BLM, which limits the usage of BLM in chemotherapy. But the underlying mechanism of such resistance is poorly understood. Here we show that the ATP binding cassette (ABC) transporter ABCC3 is required for the BLM-resistance in Arabidopsis. In a genetic screen for *ddrm* (DNA damage response mutants), we found that loss of ABCC3 confers the hypersensitivity to BLM. In contrast, overexpression of ABCC3 enhances the resistance to BLM. We further found that the expression of *ABCC3* is induced by BLM, which is dependent on the protein kinase ATM and the transcription factor SOG1, two master regulators of DNA damage response. Our study revealed that the ABC transporter contributes to BLM-resistance, indicating that the combination of ABC transporter inhibitors and BLM may enhance the efficacy of BLM in cancer therapy.

1. Introduction

Bleomycin (BLM) is a glycopeptide antibiotic originally isolated from *Streptomyces verticillis* [1]. It causes damage in DNA, RNA, cell wall and plasma membrane and is used as a chemotherapeutic agent to treat multiple cancers, including lymphomas, testicular carcinomas, and squamous cell carcinomas of the cervix, head and neck [2–4]. However, some cancer cells can develop resistance to BLM, which limits its clinical application. However, the underlying mechanism of such resistance is poorly understood. It was suggested that the resistance to BLM may be attributed to decreased drug uptake, increased drug extrusion, enhanced repair of BLM-induced DNA lesions and increased inactivation of BLM [5–7]. So far, there is no direct evidence to support that BLM could be expelled from cells by any of the known drug efflux pumps.

ATP binding cassette (ABC) transporters constitute a large protein superfamily found in all organisms ranging from bacteria to humans, and were originally characterized to transport toxic metabolites or xenobiotic compounds [8–10]. It was reported that some ABC transporters in humans could mediate multidrug resistance (MDR) to a variety of chemotherapeutic drugs, which would dramatically attenuate the efficiency of cancer pharmacotherapy [11]. Among them, P-Glycoprotein (P-gp/ ABCB1/MDR1), MRP1 and ABCG2 (BCRP) are the well-known efflux pumps to transport a wide variety of pharmacological reagents. The increased expression of these transporters in cells leads to increased efflux of drugs and decreased intracellular concentration, which is one of the major mechanisms responsible for MDR [12]. But it remains to be determined whether ABC transporters can export BLM.

In Arabidopsis, the ABCC subfamily consists of 15 members [10], and only some of these proteins were functionally analyzed. ABCC1 and ABCC2 were identified to be the major transporters of phytochelatin (PC) and contribute to detoxification of arsenic, cadmium, mercury [13, 14]. Recent studies suggested that ABCC3 can also transport PC-cadmium complexes to the vacuoles for detoxification [15]. ABCC5 functions as a transporter of phytate to affect phytate storage and regulate stomatal movement [16]. Here, we showed that the ABCC3 transporter is required for resistance to BLM in Arabidopsis. Loss of ABCC3 confers the hypersensitivity to BLM, while overexpression of ABCC3 confers BLM resistance. Furthermore, we found that BLM induced the expression of ABCC3, which is dependent on the protein kinase ATM and the transcription factor SOG1, two key regulators of DNA damage response. Thus, these findings not only reveal the new function of ABCC3 for the detoxification of xenobiotic molecules, but also provide a clue to enhance the efficacy of BLM in cancer therapy.

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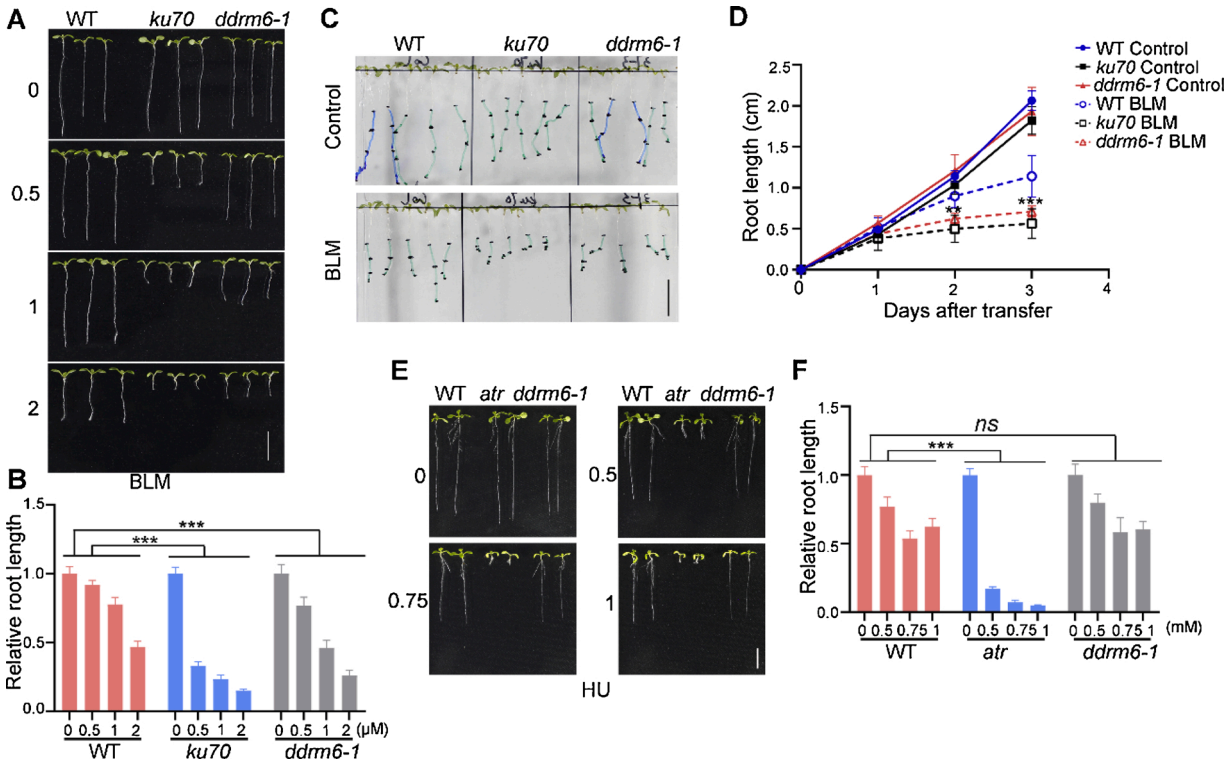

Fig. 1. The *ddrm6* mutant is specifically hypersensitive to bleomycin (BLM). (A and B) Pictures of Arabidopsis seedlings (A) and relative root length (B) of wild type (WT), *ku70*, and *ddrm6-1* grown on 1/2 MS media with different concentrations of BLM (0, 0.5, 1, 2 μM) for 7 days. (C) Pictures of seedlings transfer assay. (D) The increased root length of these plants after transfer. Five-day-old seedlings of the WT, *ku70*, and *ddrm6-1* mutant were transferred onto 1/2 MS medium with or without 2 μM BLM, and the increased root length was measured. (E and F) Pictures of Arabidopsis seedlings (E) and relative root length (F) of WT, *atr*, *ddrm6-1* grown on 1/2 MS media with different concentrations of hydroxyurea (HU) (0, 0.5, 0.75, 1 mM) for 7 days. Scale bar = 1 cm. Data represent mean ± SD (n = 10). The statistical significance was determined using Two-way ANOVA analysis. *P < 0.05, **P < 0.01, ***P < 0.001. All experiments were repeated three times with the similar results.

2. Materials & methods

2.1. Plant materials and growth condition

All *Arabidopsis thaliana* used in this study are in Columbia (Col-0) ecotype. The mutants *ku70* (SALK_123114C), *atm* (SALK_006953), *atr* (SALK_032841) and *ddrm6-2* (CS857850/WISCDSLOX481-484C11) were obtained from ABRC. The *sog1-1* mutant was described previously [17]. Primers used for genotyping are listed in Supplementary Table 1. All the transgenic lines were generated by floral-dip method [18]. Seeds were sterilized with 2% PPM and stratified at 4 °C in the dark for 2 days, and then plated on 1/2 Murashige and Skoog (MS) medium containing 1% sucrose and 0.3% phytagel. The plants were grown under long-day conditions (16 h of light and 8 h of dark) at 22 °C.

2.2. Genetic screen for ddrm

The Col-0 seeds were mutagenized with 0.2% ethyl methanesulfonate (EMS). The M1 plants were grown in soil to produce M2 seeds, which were collected from a pool of M1 plants. To screen for *ddrm*, the M2 seeds were grown vertically on 1/2 MS medium containing 2.5 μM BLM for 10 days. The plants with shorter roots than wild type under BLM treatment, but comparable to wild type under normal conditions, were considered as the *ddrm*.

2.3. Cloning of DDRM6

To clone *DDRM6*, the plants with short roots and long roots on 1/2 MS medium containing 2.5 μM BLM were separately transferred to soil and grown in a growth chamber. The genotype of each seedling was determined through examining the phenotypes of the next generation populations on 1/2 MS medium containing BLM. The leaf discs of wild type and homozygote plants were separately pooled for genomic DNA extraction, and then DNA was sequenced using Illumina HiSeq 2500 at Biomarker Technologies Corporation (China). The clean data were over 2.5 Gbp. The SIMPLE pipeline [19] was used to analyze the data to obtain candidate genes.

2.4. Vector construction

The vectors were constructed using a Lighting Cloning system (Biodragon Immunotechnology, China). For *35S:ABCC3*, the *ABCC3* genomic sequence was amplified using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, China) and was cloned into *pFGC5941* linearized with restriction endonuclease *NcoI* and *XbaI* (Thermo Fisher Scientific, USA). For *35S:ABCC3-GFP*, the ORF of *ABCC3* gene was amplified and cloned into *pFGC5941-GFP* linearized with *NcoI* and *XbaI*. For *ProABCC3:GUS* vector, the promoter (1762bp upstream of the ATG start code) of *ABCC3* was cloned into *pCAMBIA*2300-YG vector. The primers used for cloning are listed in Supplementary Table 1.

2.5. GUS staining

The plant tissues were soaked in the GUS staining solution containing X-Gluc staining solution (100 mmol/L sodium phosphate buffer pH 7.0, 0.1% Triton X-100, 0.5 mmol/L potassium ferrocyanide, 0.5 mmol/L potassium ferricyanide, and 0.5 mmol/L X-Gluc), and were infiltrated by vaccum for 15 min and then incubated at 37℃ for 12 h. After that, the samples were washed several times with 70% ethanol.

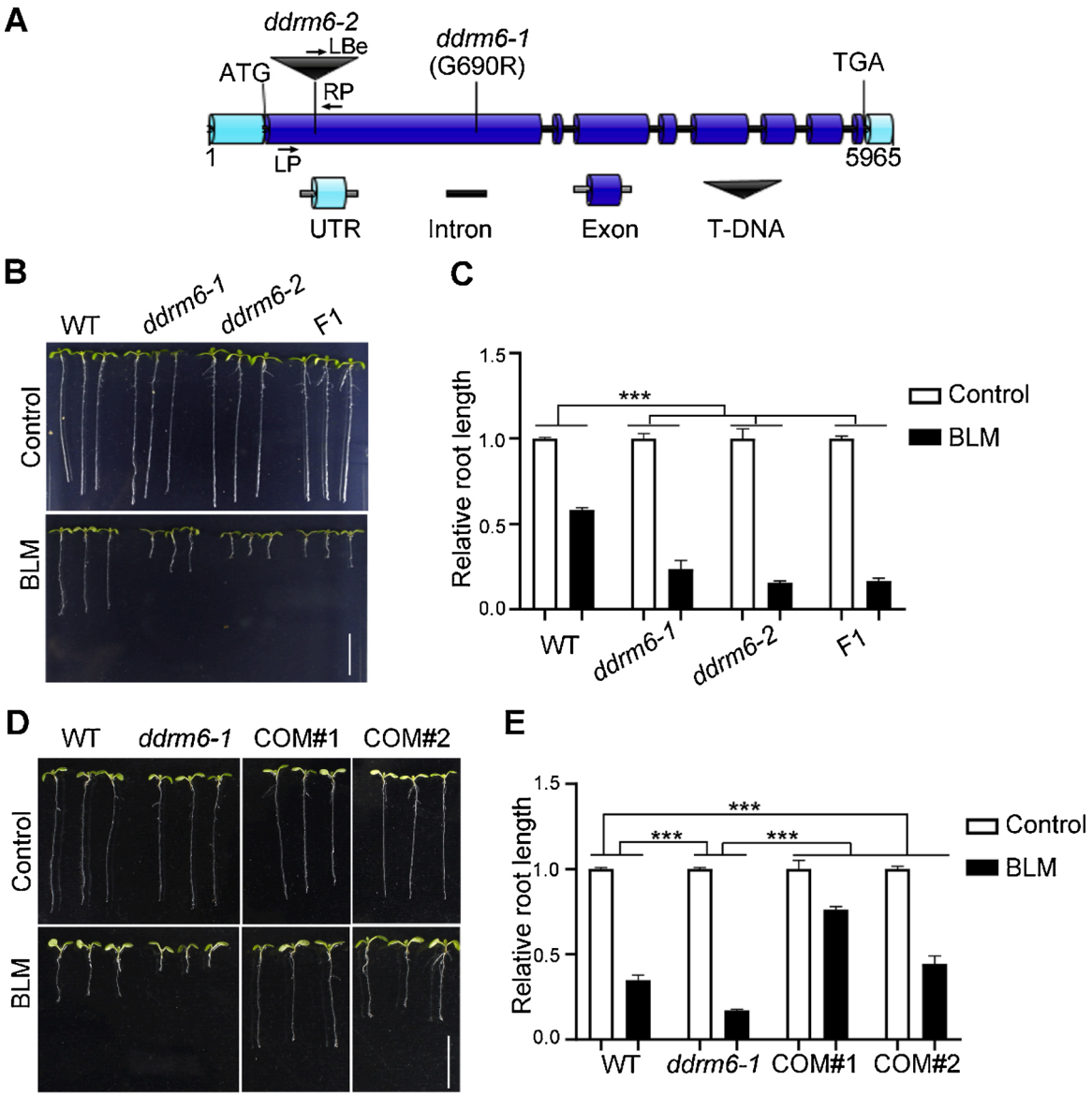

Fig. 2. Loss of function of *ABCC3* results in hypersensitivity to BLM in Arabidopsis. (A) The genomic structure of *ABCC3*. The exons are shown as dark blue boxes, and introns are represented by black lines. ATG and TGA indicate the start and stop codons, respectively. In the *ddrm6-1* mutant, Glycine (G) at the residue 690 is replaced by Arginine (R) due to the nucleotide substitution from G to A (G2552A). The T-DNA insertion site of *ddrm6-2* is shown. (B and C) Pictures (B) and relative root length (C) of WT, *ddrm6-1*, *ddrm6-2*, *ddrm6-1* X *ddrm6-2* grown on 1/2 MS medium with or without 2 μM BLM for 7 days. *ddrm6-1* X *ddrm6-2* means the F_1 seeds of *ddrm6-1* crossing with *ddrm6-2*. (D and E) Pictures (D) and relative root length (E) of WT, *ddrm6-1* and the complementation lines (COM) grown on 1/2 MS medium with or without 2 μM BLM for 7 days. The 5.4-kbp *ABCC3* genomic fragment driven by the *CaMV 35S* promoter was transformed into *ddrm6-1*. Scale bar = 1 cm. Data represent mean ± SD (n = 10). The statistical significance was determined using Two-way ANOVA analysis. *P < 0.05, **P < 0.01, ***P < 0.001. All experiments were repeated three times with the similar results.

2.6. Quantitative RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA). The reverse-transcription reaction was performed using PrimeScript RT Reagent Kit with gDNA Eraser according to the manufacturer's protocol (CoWin Biosciences, China). The quantitative PCR assays were performed on the CFX ConnecTM Real-Time PCR Detection System (Bio-Rad, USA) using MagicSYBR Mixture (CoWin Biosciences, China). The *ubiquitin 5* (*UBQ5*) was used as a reference gene. The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ method. Primers used for qPCR are listed in Supplementary Table 1.

3. Results

3.1. The ddrm6 mutant is specifically hypersensitive to BLM

BLM can induce DNA double-strand breaks (DSBs) and inhibit root growth in Arabidopsis. To dissect the mechanisms of DNA damage responses in plants, we performed a genetic screen for DNA Damage Response Mutants (*ddrm*) in Arabidopsis. The wild-type (WT) Arabidopsis seeds were mutagenized with ethyl methanesulfonate (EMS) and the M2 seeds were grown vertically on 1/2 MS medium containing BLM for 10 days. The plants with shorter roots than WT were considered as the *ddrm*. Ku70 is highly conserved in eukaryotes. It forms a heterodimer with Ku80 to mediate DSB repair. Many studies have shown that the Arabidopsis *ku70* mutant is hypersensitive to DSB-inducing agents including γ-irradiation, BLM, and methyl methane sulfonate (MMS)

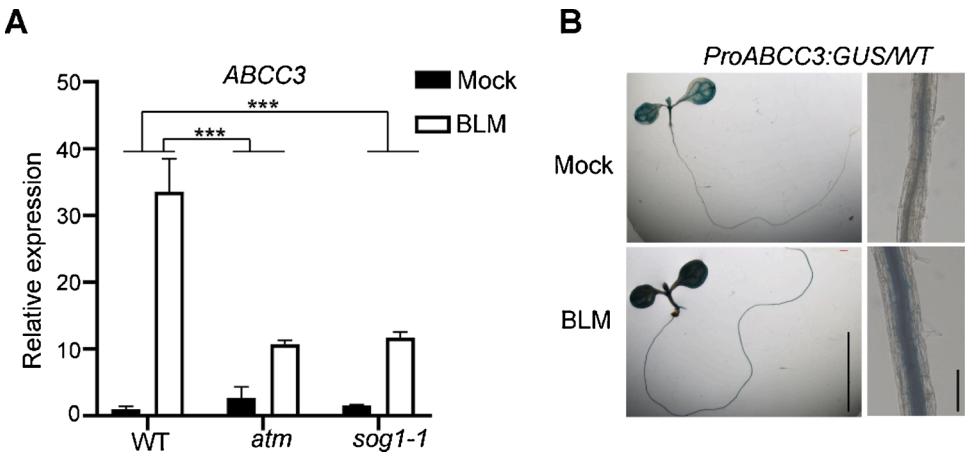

Fig. 3. The expression of *ABCC3* is induced by BLM. (A) qPCR analysis of *ABCC3* expression in WT, *atm* and *sog1-1* mutants. The 7-day-old seedlings were treatment with mock or 40 μM BLM for 4 h. Data represent mean ± SD (n = 3). The statistical significances were determined using Two-way ANOVA analysis. *P < 0.05, **P < 0.01, ***P < 0.001. (B) GUS staining of *ProABCC3:GUS* plants. The 7-day-old seedlings were treated with mock or 40 μM BLM for 12 h. The left pictures show the GUS staining of the whole seedlings and the right pictures show the GUS staining of corresponding roots. The scale bar in the left is 0.5 cm, the scale bar in the right is 0.02 mm. All experiments were repeated three times with the similar results.

[20–22]. Therefore, we used *ku70* as a positive control in our genetic screen. One of *ddrm* identified in this screen was *ddrm6-1*. As shown in Fig. 1A and B, the root length of *ddrm6-1* was comparable to that of WT and *ku70* in the absence of BLM. However, the *ddrm6-1* displayed much shorter roots than that of WT on the media with different concentrations of BLM, indicating that the *ddrm6-1* is hypersensitive to BLM. Consistent with this, the root growth of *ddrm6-1* showed stronger inhibition than that of WT after transferring from the control medium to the BLM medium (Fig. 1C and D). The response of *ddrm6-1* to hydroxyurea (HU), a chemical inducing replication stress, was comparable to WT (Fig. 1E and F), indicating that *ddrm6-1* is specifically hypersensitive to BLM.

3.2. DDRM6 encodes ABCC3

To clone the *DDRM6* gene, we performed the next-generation sequencing of DNA from both the *ddrm6-1* pool and WT pool and analyzed the sequence data using SIMPLE pipeline [19]. The analysis revealed 13256 mutation sites and one candidate gene *ABCC3* (AT3G13080) (Supplementary Table 2). In *ddrm6-1*, the G at 2068 of the coding sequence was mutated to A, resulting in Glycine to Arginine change at the residue 690 (Fig. 2A and Supplementary Table 2). G690 locates downstream of Walker A-motif and is very conserved in the ABCC subfamily (Supplementary Figs. S1 and S2), indicating that its mutation may alter the activity of ABCC transporters. To confirm that *ABCC3* was the *DDRM6* gene, we crossed *ddrm6-1* with *ddrm6-2*, a T-DNA insertion mutant of *ABCC3* (Fig. 2A and Supplementary Fig. S3). All the F_1 seedlings (F_1) were hypersensitive to BLM (Fig. 2B and C), suggesting that *ddrm6-1* and *ddrm6-2* contain the same gene mutation. To further support this, we carried out complementation test by transforming the 5.4-kbp *ABCC3* genomic fragment driven by the *CaMV 35S* promoter into the *ddrm6-1* mutant. The resulting transgenic lines (COM) were not sensitive to BLM (Fig. 2D and E), suggesting that ABCC3 can complement *ddrm6-1*. Interestingly, we found that the root lengths of these transgenic lines were even longer than that of WT on the BLM-containing medium, indicating that overexpression of ABCC3 can enhance the resistance to BLM.

3.3. Expression of ABCC3 is induced by BLM

To further investigate the role of ABCC3 in BLM detoxification, we examined the expression of *ABCC3* before and after BLM treatment through qPCR assays. We found that the expression of *ABCC3* was dramatically induced by BLM (Fig. 3A). Previous studies suggested that the protein kinase ATM and the transcription factor SOG1 play crucial roles in regulating the expression of genes involved in DNA damage response [23–25]. The induction of *ABCC3* was significantly compromised in the *atm* and *sog1-1* mutants (Fig. 3A), indicating that the expression of *ABCC3* is dependent on both ATM and SOG1. To further investigate the expression of *ABCC3* at tissue level, we generated the transgenic lines (*ProABCC3:GUS/WT*) containing the β -glucuronidase (GUS) reporter gene under the control of *ABCC3* promoter. As shown in Fig. 3B, the *GUS* gene was broadly expressed in leaves and roots, with higher expression level in vascular tissues. Consistent with the qPCR results, the GUS staining results showed that the *ABCC3* expression was strongly induced by BLM treatment (Fig. 3B).

4. Discussion

Previously, it was reported that ABCC3 functions as a transporter of glutathione-conjugates, chlorophyll catabolites and PC-cadmium complex [15,26]. Our genetic data suggested that ABCC3 is required for detoxification of BLM, thus expanding the functions of ABCC3. Since ABCC3 was identified in the proteome of the vacuolar membrane [27], it is possible that ABCC3 functions to transport of BLM from cytoplasm into vacuole. Alternatively, ABCC3 may be a transporter of glutathione (GSH)-BLM conjugates because it was reported that the concentration of GSH in cells could affect the cytotoxicity of BLM [28].

Although BLM has a wide range of cytotoxicity, DSBs are the major contributor [3,29,30]. BLM-induced DSBs can activate ATM-dependent damage response signaling [23,31,32]. In the study, we revealed that the expression of *ABCC3* is dramatically induced by BLM and this induction depends on ATM and SOG1. Thus, this finding suggests that the activated DNA damage signaling facilitates the BLM detoxification ability of cells.

Since resistance to BLM is a concern in chemotherapy, our study may have clinical implications. Through BLASTP analysis, we found that several human MRP proteins (HsMRPs) share high sequence homology to ABCC3. The sequence alignment between MRP1-4 and ABCC3 was shown in Supplementary Fig. S1. It is possible that these HsMRPs may contribute to cell resistance to BLM. If so, it is expected to enhance the efficacy of BLM in cancer treatment by a combination of HsMRPs inhibitors. Further studies are required to test these possibilities.

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Author contributions

T.L., S.X. and C.W performed research and analyzed data, S.Y. designed research, L.W. and T.L. wrote paper. L.W. agrees to serve as the

author responsible for contact and ensures communication.

Data availability statement

All the original data in this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dnarep.2021.103174.

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