

A Single Nucleotide Polymorphism in *SLC7A5* Was Associated With Clinical Response in Multiple Myeloma Patients

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Abstract. *Background/Aim:* *SLC7A5* is recognized as the major mediator of melphalan uptake into multiple myeloma (MM) cells; however, its contribution to the inter-patient variability of melphalan efficacy and toxicity is yet to be well elucidated. This study aimed to investigate the impact of a single nucleotide polymorphism (SNP) rs4240803 in *SLC7A5* on the gene expression, ex vivo sensitivity to melphalan, and clinical outcomes in MM patients who were undergoing autologous stem cell transplantation with high-dose melphalan. *Materials and Methods:* Peripheral blood mononuclear cells (PBMC) were collected from 108 MM patients prior to melphalan therapy. Clinical data were also collected from these patients following melphalan therapy. *Results:* rs4240803 was associated with elevated expression of *SLC7A5* mRNA, higher ex vivo sensitivity to melphalan in PBMCs, and positive 90-day response in these patients ($p=0.047$, 0.10 , 0.049 , respectively). *Conclusion:* rs4240803

impacted the expression of *SLC7A5*, thus contributing to the clinical response of MM patients to melphalan therapy.

Multiple myeloma (MM) affects approximately 83,000 US citizens and over 50% of patients die within 5 years of diagnosis. Autologous hematopoietic stem cell transplants (AutoHSCT) in MM improves overall survival by 1 year on average (1-4) and remains the standard of care for fit MM patients despite the increasing availability of novel agents, such as proteasome inhibitors, immunomodulatory drugs, and monoclonal antibodies. However, the treatment outcome of autoHSCT varies considerably among MM patients, and about 20% of patients exhibit melphalan-resistant myeloma, as demonstrated by a progression-free survival (PFS) of less than 12 months, much shorter than the PFS of the majority of MM patients (3, 4). Recent studies indicate that a variety of mechanisms, such as the influx and efflux of melphalan into cells (5, 6), DNA repair of melphalan-induced damage (7, 8), and melphalan metabolism/elimination (9, 10), may influence the patient's response to melphalan.

SLC7A5 (also known as LAT1, L-type amino acid transporter 1) is a Na⁺ and pH-independent neutral amino acid transporter (11, 12). In association with *SLC3A2* (also designated as CD98 or 4F2hc, a small type II membrane glycoprotein), *SLC7A5* forms a heterodimeric transmembrane protein complex that catalyzes the transport of large neutral amino acids, such as leucine, phenylalanine, tyrosine, and histidine (11, 13-16). It has been reported that melphalan, a bis(2-chloroethyl) amino derivative of L-phenylalanine, is recognized and transported into cells by *SLC7A5* (6, 17, 18). While down-regulation of *SLC7A5* expression has been found to be associated with melphalan

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uptake in different MM cell lines (6), a single nucleotide polymorphism (SNP) in the first intron of *SLC7A5* (rs4240803, a G→A transition) was associated with the need of total parenteral nutrition (TPN; an indicator of melphalan-caused mucosal injury) in a cohort of MM patients after autoHSCT therapy (9). These results suggest that *SLC7A* may influence the cellular intake of melphalan as well as the toxicity of melphalan in MM patients. This premise has been further supported by our recent pharmacokinetic (PK) modeling study showing that *SLC7A5* rs4240803 might influence melphalan distribution within the peripheral compartment, thus contributing to the interpatient variability in melphalan PK properties (10). However, whether and how rs4240803 impacts the pharmacodynamics (PD) of melphalan, especially the efficacy and the toxicity in MM patients is unknown.

The aim of our study was to evaluate the potential correlation of rs4240803 with *SLC7A5* expression and clinical outcomes, namely, 90-day response, progression-free survival (PFS), and the severity of oral mucositis in MM patients after autoHSCT.

Materials and Methods

Peripheral blood mononuclear cell (PBMC) samples. Prior to melphalan infusion, samples of venous blood were obtained from 108 MM patients who underwent melphalan-based autologous stem cell transplantation as part of a prospective clinical trial were procured following approval from the Cancer Institutional Research Board's guidance (NCT01653106) (10). PBMCs were separated using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) at The Ohio State University Leukemia Tissue Bank as previously described (10).

Genotyping and gene expression assays. DNA and RNA were purified from PBMCs using a Blood DNA/RNA Purification kit (Qiagen, Valencia, CA, USA). cDNAs were synthesized using a High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA). Genotypes of *SLC7A5* rs4240803 were determined on a QuantStudio™ 7 Flex system (Thermo Fisher Scientific, Waltham, MA, USA) using a pre-validated TaqMan genotyping assay: C_1228783_10 (Thermo Fisher Scientific). Assays were conducted in duplicates.

The mRNA expression levels of target genes in MM cells and PBMCs were quantitatively assessed using TaqMan gene expression assays (Life Technologies) using the following inventoried primer/probes: Hs01001189_m1 (solute carrier family 7 member 5, *SLC7A5*), Hs00374243_m1 (solute carrier family 3 member 2, *SLC3A2*); Hs99999905_m1 (glyceraldehyde 3-phosphate dehydro-genase, *GAPDH*). *GAPDH* was used as an endogenous reference for normalized gene expression. Each gene was amplified separately, and all experiments were performed in triplicate. The relative expression level (REL) of a target was determined using a comparative C_q method in which REL was defined as 2^{-ΔC_q} (19). Of note, the expression level of a target gene presented in this study should be regarded as the baseline expression level of that specific target gene in PBMCs prior to melphalan treatment.

Ex vivo cell viability assays. PBMCs were seeded at 5000 cells/well in 96-well plates in 100 μl of complete medium containing Advanced RPMI1640 (Life Technologies Corporate) supplemented with 5% fetal bovine serum (FBS; Life Technologies,) and 1×Antibiotic Antimycotic solution (Sigma-Aldrich, St Louis, MO, USA). Cells were grown at 37°C in a 90% humidified environment in 5% CO₂ overnight. Subsequently, cells were incubated in complete medium containing various concentrations of melphalan (Sigma-Aldrich, St Louis, MO, USA) for another 24 h. Cell growth was assayed using a WST-1 Cell Proliferation Assay Kit (Roche, Indianapolis, IN, USA). Assays were performed in triplicate. Absolute IC₅₀ values (the concentration of melphalan required to inhibit 50% of the cell viability) were determined using Kaleidagraph software (Synergy Software, Reading, PA, USA) as previously described (10).

Pharmacokinetic (PK) parameter determination. Venous blood samples were collected in heparin tubes prior to melphalan administration (time 0) and then at designated time points following completion of melphalan infusion: 5, 30, 45, 60, 180, and 360 min. The plasma concentration of melphalan was assessed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (10). Non-compartmental PK parameters, such as AUC_{inf}, C_{max}, T_{max}, were determined using Phoenix WinNonlin (v6.3, Pharsight, Mountain View, CA, USA).

Statistical analyses. All statistical analyses were conducted using R3.3.2 (R Foundation for Statistical Computing; cran.r-project.org). For rs4240803, the consistency between its distribution and the Hardy Weinberg equilibrium (HWE) principle was analyzed using chi-square test. Count data were analyzed using Fisher's exact tests or chi-square tests.

Gene expression data were subjected to log transformation to fulfill the normality requirement. Group wise differences in gene expression were analyzed using unpaired two-sample Student's *t*-tests where appropriate. The correlations between *SLC7A5* and *SLC3A2* gene expression, between *SLC7A5* gene expression and *ex vivo* IC₅₀ values were evaluated and tested using Pearson's product-moment correlation coefficients (19).

Time to relapse was determined as the time from transplantation until the earliest of the following time points: progressive disease, clinical relapse, or relapse from CR (complete response) as determined by the International Myeloma Working Group (IMWG). Patients without known progression were censored at the date of last follow up. Time to relapse was calculated from Kaplan–Meier curves with the difference between the curves analyzed using the log-rank test.

The potential association between mucositis (no: mucositis grade 0 and 1; yes: mucositis grade 2 and 3) and SNP as well as other covariates, between response at day 90 after transplantation (poor response: negative, minor response and partial response; good response: very good partial response, complete response, and stringent complete response) and SNPs as well as other covariates were evaluated using logistic regression.

p-Values were two-sided, and unless specified, values of *p* < 0.05 were regarded as statistically significant.

Results

***SLC7A5* rs4240803 in PBMCs.** The majority of patients were Caucasian (95/108, 88.0%), such that the minor allele frequency (MAF=0.296) is comparable with the previously

Table I. Demographics and clinical characteristics of patients in the current study.

Characteristics	SLC7A5 rs42420803 GG (n=53)	SLC7A5 rs42420803 AG/AA (n=55)	p-Value**
Age (years)			
Median (range)	60 (35-70)	59 (37-72)	0.89
Gender			0.57
Female	26 (49.1%)	23 (41.8%)	
Male	27 (50.9%)	32 (58.2%)	
Race			
White	45 (84.9%)	50 (90.9%)	0.43
Other	8 (15.1%)	5 (9.1%)	
Melphalan dose*			0.23
140 mg/m ²	6 (11.3%)	12 (21.8%)	
200 mg/m ²	47 (88.7%)	43 (78.2%)	
CrCL (ml/min)			0.64
CrCL <60	8 (15.1%)	11 (20%)	
CrCL ≥60	45 (84.9%)	43 (78.2%)	
		(1 missing)	
AUCinf (mg*min/l)			
Median (range)	878 (336-1640)	871.5 (388-1580)	0.92
Risk			0.18
Standard risk	35 (66.0%)	25 (45.5%)	
Intermediate/high risk	17 (32.1%)	27 (49.1%)	
	(1 missing)	(3 missing)	
Mucositis			0.96
0	19 (35.8%)	21 (38.2%)	
1	25 (47.2%)	24 (43.6%)	
2	8 (15.1%)	7 (12.7%)	
3	1 (1.9%)	2 (3.6%)	
		(1 missing)	
Response at Day 90 post-transplantation***			0.07
NE/MR/PR	23 (43.4%)	14 (25.5%)	
VGPR/CR/sCR	17 (32.1%)	24 (43.6%)	
	(13 missing)	(17 missing)	
Length of stay in hospital (days)			
Median (range)	14 (11-21)	14 (10-21)	0.81

Sd: Standard deviation; CrCL: creatinine clearance. *Melphalan dose adjusted by the patient's BSA (body surface area, m²). **All count data were analyzed using Chi square tests or Fisher's exact tests where appropriate, and continuous data were analyzed using U rank sum tests. All tests were two-sided. Sd: Standard deviation. Missing data were not included in the statistical analyses. ***For 90-day response, NE: Not evaluable; MR: minor response; PR: partial response; VGPR: very good partial response; CR: complete response; sCR: stringent complete response.

reported MAF (0.282) in a Caucasian population (9). The genotype frequencies of rs4240803 are in Hardy-Weinberg equilibrium ($p=0.99$). A total of 53 patients carried the wild type genotype GG, 46 patients were heterozygous GA carriers; and 9 patients harbored the homozygous AA variant genotype. Table I summarizes the demographic and clinical

characteristics of these patients. There is no statistically significant association between rs4240803 GG and GA/AA carriers with age, sex, race (white, other), risk (standard, intermediate/high), melphalan dose (140 mg/m², 200 mg/m²), creatinine clearance (CrCL <60 ml/min, CrCL ≥60 ml/min), AUC (area under the concentration-time curve, mg*min/l), length of stay, and oral mucositis score (all p -values >0.15).

Rs4240803 is located within the first intron of the *SLC7A5* gene in the proximity of a putative transcription enhancer (9). It is anticipated that rs4240803 may impact the transcription of *SLC7A5* rather than directly the *SLC7A5* protein. To address this premise, we evaluated the mRNA expression of *SLC7A5* in PBMCs from patients with different rs42420803 genotypes. As shown in Figure 1A, the expression level of *SLC7A5* mRNA was significantly associated with rs42420803 genotype ($p=0.047$). The expression level of *SLC7A5* mRNA in patients with rs4240803 A* variant alleles (AG/AA) was higher than that in patients homozygous GG at rs4240803. Since *SLC7A5* functions as a heterodimer with a covalently-bound *SLC3A2* unit (through a disulfide bond) (20, 21), the expression level of *SLC3A2* mRNA was also evaluated in these PBMC samples. Overall, there is a strong positive correlation between *SLC7A5* and *SLC3A2* in mRNA expression (Pearson's correlation coefficient=0.46, $p<0.001$) (Figure 1B). Since *SLC7A5*, in association with *SLC3A2*, functions in the uptake of melphalan in MM cells, we anticipated that elevated *SLC7A5* expression in PBMCs from patients with rs4240803 AG/AA genotypes would enhance the uptake of melphalan and sensitize these cells. Results from our *ex vivo* cytotoxicity assays support this premise. As shown in Figure 1C, PBMCs with higher *SLC7A5* mRNA expression tended to have lower IC₅₀ values of melphalan ($p=0.10$), indicating that these cells were more sensitive to melphalan. This observation was consistent with previous findings that as a melphalan transporter, down-regulation of *SLC7A5* expression by siRNA led to reduced intake of melphalan in MM cell lines (6).

SLC7A5 rs42420803 and clinical outcomes in MM patients. Subsequently, the potential association between rs4240803 and 90-day response in these MM patients was investigated. As shown in Table I, 63.1% (24/39) of patients with 90-day response data and rs42420803 AA/AG had "good" 90-day responses (including very good partial response, complete response, and stringent complete response), considerably higher than the response rate 42.5% ($p=0.07$) of rs42420803 GG patients. Univariate logistical regression analyses showed that rs4240803, melphalan dose, and renal function (CrCL) were associated with 90-day response in these MM patients. The odds ratio (OR) of having 90-day response between rs4240803 AA/AG and GG was 2.32 (AA/AG vs. GG, 95%CI=0.94-5.87, $p=0.07$); OR between the various melphalan dose levels was 3.49 (200 mg/m² vs. 140 mg/m², 95%CI=0.92-17.0, $p=0.08$); OR between the various CrCL

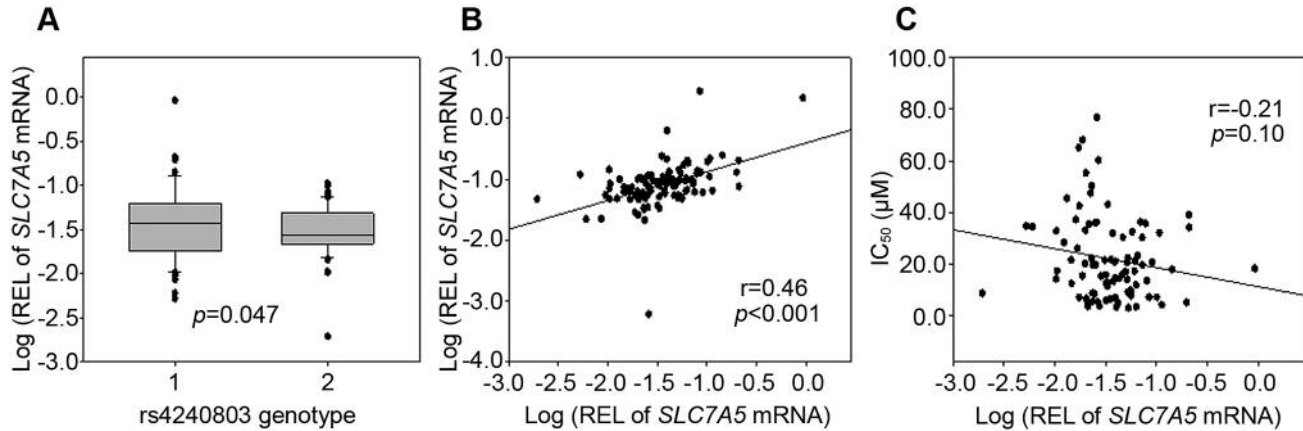


Figure 1. Association of *SLC7A5* rs4240803 genotypes (AA/AG, GG) with gene expression and ex vivo sensitivity to melphalan in PBMCs from MM patients. A) Box plots to analyze the expression of *SLC7A5* mRNA in PBMCs from MM patients with different *SLC7A5* rs4240803 genotypes (AA/AG, GG). qRT-PCR-based assays using pre-validated primers and probes were used to determine the mRNA expression levels of target genes. GAPDH was used as an endogenous reference, and the relative expression level (REL) of a target gene was defined using the $2^{-\Delta\Delta C_t}$ method (19). Each assay was conducted in triplicate and the average value was used for statistical analyses. All expression data were subjected to log transformation to fulfill the normality requirement, and two-sided Student's t-test was used to analyze the difference in *SLC7A5* expression between patients with different rs4240803 genotypes: 1, AA/AG; 2, GG. B) Scatter plots illustrating the correlation between *SLC7A5* and *SLC3A2* in mRNA expression in PBMCs from MM patients. The potential correlations were analyzed using Pearson's correlation tests. *r*, Pearson's correlation coefficient. C) Scatter plots illustrating the correlation between *SLC7A5* mRNA expression and IC₅₀ values of melphalan in PBMCs from MM patients. IC₅₀ values were evaluated using WST-1 cell proliferation assays as previously described. (10). Assays were performed in triplicate, and the average of three IC₅₀ values was used in the statistical analyses.

levels (<60 ml/min vs. ≥60 ml/min, 95%CI=0.87-11.9, $p=0.09$). In contrast, the association between 90-day response and age, sex, risk, AUC_{inf} were insignificant ($p>0.2$). With adjustments by melphalan dose and CrCL, OR between *SLC7A5* rs4240803 AA/AG and GG was 2.70 (95%CI=1.02-7.69, $p=0.049$). *SLC7A* rs4240803 (G→A) favored 90-day response in MM patients after autoHSCT. Further analyses revealed a marginally significant association between 90-day response and the expression level of *SLC7A5* mRNA in the cohort of MM patients in our study ($p=0.15$) and patients with higher *SLC7A5* mRNA expression trended to have higher odds of “good” 90-day response. These results are consistent with a previous study showing that the overall response rate in MM patients with high *SLC7A5* protein expression was significantly higher than that in patients with low *SLC7A* protein expression (60% vs. 17.6%, $p=0.03$) (21).

Furthermore, we investigated the potential association of rs4240803 with PFS or the severity of oral mucositis. Our results did not show any significant association for either comparison ($p=0.66$, 0.97 , respectively). Interestingly, results from a previous study indicated that rs4240803 was significantly associated with TPN requirement in MM patients, a global measure of gastrointestinal toxicity (9). Compared with patients carrying rs4240803 GG, patients with rs4240803 A* alleles had lower odds of IPN use (OR=0.45, 95%CI=0.25-0.79, $p=0.007$), indicating that

rs4240803 favored lower gastrointestinal toxicity. Notably, TPN requirement was used as an index of gastrointestinal toxicity encompassing clinical manifestations including pain, nausea, and diarrhea. Therefore, it served only as a proxy for chemotherapy-induced mucositis due to lack of formal mucositis scores for the majority of studied patients (9).

Discussion

SLC7A5 and *SLC7A8* are L-type amino acid transporters involved in the passage of large neutral amino acids such as leucine, isoleucine, phenylalanine, tyrosine, tryptophan, methionine, histidine, and valine into the cell (5). While *SLC7A8* is expressed primarily in the kidneys and bowel (22), *SLC7A5* is widely expressed in all human tissues except intestine (13). As such, *SLC7A5* is assumed to play a more important role than *SLC7A8* in amino acid intake in dividing cells (6). Elevated expression of *SLC7A5* has been found in a variety of human cancer cells and tumors, including lung, brain, prostate, stomach, and pancreatic cancers (21). It is generally believed that the expression levels of *SLC7A5* are relevant to tumor development, angiogenesis, and poor prognosis in cancer patients (9, 21). In multiple myeloma, *SLC7A5* and *SLC7A8* are regarded as the major mediators of melphalan uptake in cells, due to the high resemblance between melphalan and phenylalanine in molecular structure and the binding affinity to *SLC7A5* (5).

While it has been found that melphalan competes with endogenous large neutral amino acids for SLC7A5 binding, transcriptional knockdown of *SLC7A5* by siRNA led to decreased intake of melphalan in MM cells, indicative of the crucial roles of SCL7A5-mediated transport in the absorption and distribution of melphalan (6). Furthermore, Isoda *et al.* (21) reported that MM patients who have a higher SLC7A5 protein expression were more likely to have a positive clinical response after treatment with melphalan and prednisolone, implying that the functions of SLC7A5 could influence the clinical response in MM patients. In the present study, we quantitatively evaluated the impact of rs4240803 on the expression of *SLC7A5* mRNA and the sensitivity of PBMCs to melphalan in 108 MM patients with standard autoHSCT-HDM treatment. rs4240803 was significantly associated with increased *SLC7A5* mRNA expression in PBMCs, resulting in greater sensitization of these cells to melphalan cytotoxicity. Moreover, rs4240803 was associated with a higher possibility to have a positive 90-days response in these patients. To our knowledge, this is the first report on the association of *SLC7A5* rs4240803 with a positive clinical response in MM patients.

Our study has also provided novel insights into understanding the mechanisms underscoring the observed association between rs4240803 and clinical response. Even though up-regulation of SLC7A5 at mRNA and protein levels has been previously found in different human cancers, including multiple myeloma (21), little is known about the cis- and trans-regulatory factors that control transcription of *SLC7A5*. The proximity of rs42400803 to a putative enhancer segment in the first intron of *SLC7A5* (9) provides a rationale for predicting a functional role in modifying transcription of *SLC7A5*. Our data showed that rs42400803 correlated with enhanced transcription of *SLC7A5* in PBMCs. This is the first report on the molecular mechanisms underlying the impact of rs4240803 in MM patients. Because of the well-documented similarities in the molecular characteristics between PBMC and bone marrow plasma cells (BMPC) from the same patient, we anticipate that rs4240803 could up-regulate the transcription of *SLC7A5* in BPMCs as well, subsequently increasing the uptake of melphalan into these tumor cells. Higher intake of melphalan would enhance the cytotoxicity of melphalan in BPMCs and consequently lead to better clinical response in MM patients carrying this variation.

It is important to note that multiple molecular mechanisms may be involved in the regulation of SLC7A5 at the transcriptional and post-transcriptional levels, which would ultimately influence the functioning of SLC7A5 in MM patients. First, it has been found that *SLC7A5* is over-expressed in many human cancers with an increased demand of amino acids, suggesting that the expression of *SLC7A5* might be inducible (5, 16). Second, there is a

considerable number of SNPs identified in the coding and non-coding regions of *SLC7A5* that may impact the transcription of *SLC7A5* individually or in combination (9, 10, 18). While rs4240803 was found to be associated with the gastrointestinal toxicity and the distribution of melphalan in the peripheral compartment in two different cohorts of MM patients, respectively (9, 10), a group of 44 polymorphisms in *SLC7A5* (excluding rs42420803) were identified in a previous study including 64 MM patients (18). These 44 SNPs were located in coding/non-coding regions other than intron 1, including 5' flank region, exon 1, and introns 2–7, and none of them were found to be significantly associated with the expression of *SLC7A5* mRNA. However, we cannot rule out the possibility that the impact of rs4240803 on *SLC7A5* transcription is influenced by some of these additional *SLC7A5* SNPs through linkage-equilibrium or other molecular mechanisms, *i.e.* some of these SNPs might have a bearing on *SLC7A5* transcription in the presence of rs4240803. Third, it has been reported that different amino acids (as well as melphalan) compete with each other for binding to SLC7A5 in cells (14, 23, 24). As such, *SLC7A5*-mediated transport of melphalan in cells could be impacted by certain amino acids (such as glutamine, histidine, leucine) and the action of amino acid transporters other than SLC7A5. Taken together, these observations suggest that the putative impacts of rs4240804 on *SLC7A5* transcription and subsequent functioning in MM patients could be “cofounded” with effects caused by other SLC7A5-relevant or irrelevant molecular mechanisms/pathways. Arguably, rs4240803, like polymorphisms in most drug metabolism-related genes, would exert a moderate or even “small” effect on melphalan metabolism and functioning in MM patients (25), hence, meta-analyses and more extensive analyses in large cohorts of MM patients in controlled clinical trials would be essential to elucidate the contribution(s) of this and other *SLC7A5* SNPs to the interpatient heterogeneity of melphalan response. The relatively small size (N=108) of the patient population and the unavailability of clinical data (notably for 90-day response) from a considerable portion of patients limited the full testing of some of our hypotheses and conservatively restricted our ability to demonstrate definitive outcomes. Nonetheless, our results strongly support further studies to elucidate the potential roles of *SLC7A5* rs4240803 (and SLC7A5 in general) in MM patients with autoHSCT-HDM therapy.

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