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A Single Nucleotide Polymorphism (SNP) in the *SLC22A3* Transporter Gene Is Associated With the Severity of Oral Mucositis in Multiple Myeloma Patients Receiving Autologous Stem Cell Transplant Followed by Melphalan Therapy

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Abstract. *Background: It has been reported that expression of OCT3 enhanced the sensitivity to melphalan in cells, indicative of potential roles of OCT3 in melphalan transport. Herein we investigated the association of select single nucleotide polymorphisms in SLC22A3 (gene encoding OCT3) with clinical outcomes in multiple myeloma (MM) patients with hematopoietic autologous stem cell transplants followed by high-dose melphalan therapy. Materials and Methods: Melphlan concentrations in blood samples from 108 MM patients were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS); genotypes of rs2048327, rs1810126, and rs3088442 in these patients were determined using quatitive RT-PCR assays. Results: Rs3088442 A variant-carriers had a significantly increased risk of severe oral mucositis in comparison with homozygous rs3088442 G-carriers with adjusted odds ratio of 4.00 (95% CI=1.25-14.7; p=0.027). Rs3088442 A carriers tended to have lower creatinine clearance (p=0.10) and higher maximum plasma concentration of melphalan (p=0.07). Conclusion: OCT3 might be involved in melphalan transport in MM patients.*

This article is freely accessible online.

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Key Words: Multiple myeloma, melphalan, OCT3, *SLC22A3*, oral mucositis.

Multiple myeloma (MM) represents the 2nd most common hematological malignancy characterized by a pattern of recurrent relapses and remains incurable due to resistance and relapse in almost all patients (1). Hematopoietic autologous stem cell transplant (HSCT) with high-dose intravenous melphalan (HDM) remains the "gold" standard of care and the most effective treatment for transplant-eligible patients (2-5). Notably, there are considerable variations among MM patients in regard to the efficacy of autoHSCT-HDM therapy and melphalan-induced side-effects. On one hand, about 20% of patients exhibit resistance to melphalan, as demonstrated by progression-free survival (PFS) much shorter than the PFS of the majority of MM patients, which is partially ascribed to inadequate dosing (6, 7). On the other hand, some patients suffered from severe adverse effects, such as oral mucositis, gastrointestinal toxicity, and infection, which arguably result from excessive dosing (8-12). Since the standard 200 mg/m^2 of melphalan is used in most MM patients (for some patients with severe renal dysfunction, 140 mg/m^2), the ability for melphalan to be metabolized in patients appears to be closely related to clinical outcomes (responses and adverse effects) (8, 11). To date, it has been known that hydrolysis and renal

excretion are the major metabolic pathways in MM patients (13, 14). Hydrolysis of melphalan is a spontaneous process and does not require enzymatic activation of melphalan. This process occurs quickly in the plasma after melphalan administration, thus being arguably regarded as the main metabolic pathway. Interestingly, it has been found that up to 40% of plasma melphalan is excreted intact in urine in MM patients (13). Even though transporter-mediated passive transport is generally believed to be the mechanism underlying renal excretion of melphalan (15-17), knowledge on these transporters and their function in melphalan metabolism remains limited.

Renal drug excretion in proximal tubules is mediated by a group of transporters with broad specificities, namely the solute carrier 22 (SLC22) transporter family (18-21). This family includes organic anion transporters, such as OAT1 (*SLC22A6*), OAT3 (*SLC22A8*), and organic cation transporters 1, 2, and 3 (OCT1-3, *SLC22A1-3*). While OATs take up a multitude of negatively charged pharmaceuticals from the blood into proximal tubule cells (18, 21), the universal negative membrane potential in cells facilitates OCTs-mediated cellular uptake of organic cation substrates, including a broad spectrum of toxins, endogenous compounds, and drugs (such as serotonin, acyclovir, metformin, oxaliplatin, and cisplatin). Therefore, OCTs may influence the pharmacokinetic and pharmacodynamics properties of these drugs as well as the patients' response (18-20).

Despite their similarities in structure and transport function, the impact of OCT1, OCT2, and OCT3 on specific drugs vary greatly, which is arguably ascribed to their differential expression patterns in tissues (18, 22, 23). OCT1 is mainly expressed in the liver, and mediates uptake and accumulation of drugs in the liver; OCT2 is highly expressed in the kidney, and its correlation with deposition and renal clearance of many drugs has been observed (22, 23). By virtue of their interactions with a wide variety of drugs, OCT1 and OCT2 have been included in the current FDA and EMA guidances for drug-drug interactions (24). In comparison, it is presumed that OCT3 is ubiquitously expressed with the highest *SLC22A3* mRNA levels found in the kidney and liver (22, 23). Since its broad substrate profile is overlapping with those of OCT1 and OCT2, OCT3 has been long regarded as a less important contributor to uptake than OCT1 in the liver or OCT2 in the kidney (22, 23). Recent studies have shown that *SLC22A3* knockout led to reduced metformin bioavailability and attenuated pharmacological response to this drug in mice (with intact *OCT1* and *OCT2*), indicating that OCT3 may contribute to metformin disposition in a way independent of OCT1 and OCT2 (25). This finding is further supported by a study showing that a 3'-UTR variant of *SLC22A3* down-regulated *SLC22A3* expression and was associated with reduced metformin response in humans. More interestingly, it has been shown that OCT3 is localized at both the basolateral (blood-facing) and apical (saliva-facing) membranes of salivary gland acinar cells, suggesting a dual role of OCT3 in mediating both uptake and efflux of organic cations in the salivary glands (26, 27). OCT3 has also been found to be localized at the sinusoidal membrane of hepatocytes, the basolateral membrane of renal proximal tubule epithelial cells, and the luminal membranes of bronchial epithelial cells and small intestinal enterocytes, indicating that the cellular orientation of OCT3 is tissue-specific and its roles in intake and efflux of specific drugs might also be tissue-specific (19, 20, 27-30). Additionally, a number of single nucleotide polymorphisms (SNPs) in coding and non-coding regions of the *SLC22A3* gene have been found to contribute to inter-individual variations in cationic drug position in patients (22, 28, 29). As for melphalan, Shnitsar and his colleagues showed that elevated expression of *SLC22A3* enhanced the sensitivity to melphalan in selected kidney carcinoma cell lines (30). Taken together, these results suggest that OCT3 or SNPs in the *SLC22A3* gene might be involved in cellular uptake and tissue disposition of melphalan, thus impacting its efficacy and/or toxicity in MM patients.

In this study, we investigated the prevalence of three SNPs in *SLC22A3*, rs2048327, rs1810126, rs3088442, in a cohort of 108 MM patients with autoHSCT-HDM therapy and their potential associations with clinical outcomes in these patients, namely, 90-days response, relapse-free survival, and the severity of oral mucositis.

Materials and Methods

Peripheral blood mononuclear cell (PBMC) samples. PBMCs from 108 MM patients who underwent melphalan-based autologous stem cell transplant (prior to melphalan treatment) as part of a prospective clinical trial were procured following approval from the Cancer Institutional Research Board's guidance (IRB#2011C0080, NCT01653106) (5). DNA and RNA were purified using a Blood DNA/RNA Purification kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. cDNAs were synthesized using a High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA).

Cell lines. Multiple myeloma (MM) cell lines, MM1S, NCI-H929 (hereafter, H929), and U266 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). LP1 was a generous gift from Dr. Falvia Pichiorri, The Briskin Myeloma Center, (City of Hope, CA, USA). Cells were maintained in advanced RPMI1640 (Life Technologies), supplemented with 5% fetal calf serum (FBS; Life Technologies) and 1×Antibiotic Antimycotic solution (Sigma-Aldrich, St Louis, MO, USA), and were grown at 37˚C and 5% CO_2.

Oral cell lines, CAL27, SCC4, and SCC22A were purchased from ATCC. SCC83-82, a precancerous oral cell line, was kindly provided by Dr. Christopher Weghorst at the College of Public Health, The Ohio State University (31). Cells were grown in advanced DMEM/F12 (Life Technologies Corporate), supplemented with 5% FBS, and 1×Antibiotic Antimycotic solution as previously described (32).

HEK293 and HEK293_OCT3, a transfected HEK293 cell line with stably expressed *SLC22A3*, were gifts from Dr. Alex Sparreboom at College of Pharmacy, The Ohio State University. Cells were grown under the same conditions as the other cell lines except that advanced DMEM with high glucose media (Life Technologies Corporate) was used for these two cell lines, and G418 (1,000 μg/ml, Life Technologies) was included in the media for HEK293_OCT3.

All cell lines were regularly authenticated using short tandem repeat polymorphism (STRP) analysis as recommended by ATCC and were mycoplasma free. Cellular experiments were conducted using cells with less than 20 passages after thawing.

SNP genotyping. Genotypes of three selected SNPs in 3' UTR of *SLC22A3* were determined on a QuantStudio™ 7 Flex system (Thermo Fisher Scientific, Wakthan, MA, USA) using the following Taqman® pre-validated genotyping kits (Life Technologies): for *SLC22A3* rs2048327, C__2737090_10; for *SLC22A3* rs1810126, C__11326397_10; for *SLC22A3* rs3088442, C__2763387_10. Assays were conducted in duplicate (5).

Gene expression. The expression levels of target genes in PBMCs and MM cells were quantitatively assessed using Taqman® gene expression assays (Life Technologies) using the following inventoried primer/probes: for *SLC22A3,* Hs01009568_m1 and Hs01009571_m1; for *SLC7A5,* Hs01001189_m1; for *SLC3A2*, Hs00374243_m1; for *GAPDH* gene (glyceraldehyde 3-phosphate dehydrogenase, 4333764-0805024). *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 Cq method in which REL was defined as $2^{-\delta Cq}$. Notably, the expression level of a target gene in PBMCs presented in this study should be regarded as the baseline expression level since RNA and DNA samples were extracted from PBMCs without any *in vivo* and *ex vivo* melphalan treatment (5, 32).

Cell viability. Different cells were seeded at 2,000 cells/well in 100 μl of corresponding media and incubated at 37˚C and 5% CO_2 overnight. Subsequently, cells were incubated with media containing various concentrations of melphalan (Sigma-Aldrich, St Louis, MO, USA) for another 72 h. Cell viability was assayed using WST-1 Cell proliferation Assay kit (Roche, Indianapolis, IN, USA) following the manufacturer's directions. Assays were performed in triplicate at least twice. Absolute IC_{50} values (the concentration of melphalan required to inhibit 50% of the cell viability) were determined using Kaleidagraph software (Synergy Software, Reading, PA) as previously described (32).

Pharmacokinetic (PK) parameter determination. Following the aforementioned IRB-approved procedure, venous blood samples were collected in heparin tubes prior to melphalan administration (time 0) and then at different time points after completion of melphalan infusion: 5, 30, 45, 60, 180, and 360 min. The concentration of melphalan in each plasma was assessed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following a well-validated procedure (33). 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 R3.4 (R Foundation for Statistical Computing; https://CRAN.R-project.org). Discrete data were analyzed using Fisher's exact tests or χ^2 tests where appropriate; continuous data were analyzed using Student's *t*-tests. For each SNP, the consistency between its distribution and the Hardy Weinberg equilibrium (HWE) principle was analyzed using χ^2 test. Potential nonrandom allele associations between selected SNPs were investigated using χ^2 tests.

Gene expression data were subjected to log transformation to fulfill the normality requirement (5). Group-wise differences in gene expression were analyzed using unpaired/paired two-sample Student's *t*-tests where appropriate.

Univariate and multivariate logistic regression analyses were used to investigate the potential associations between *SLC22A3* SNPs and the incidence of mucositis (no: mucositis grades 0/1; yes: mucositis grades 2/3) or the incidence of response at day 90 after transplant (no: minor response and partial response; yes: very good partial response, complete response, and stringent complete response).

Time to relapse was defined as the time from transplant (Time 0) 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. The median time to relapse was calculated from Kaplan-Meier (KM) curves, and the difference between the KM curves was analyzed using the log-rank test.

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

Results

Genotyping of rs2048327, rs1810126, and rs3088442 in SLC22A3 gene. Encoded by the *SLC22A3* gene, OCT3 participates in the cellular uptake and elimination of various cationic substrates, including therapeutically important agents as well as in the activation of biogenic amines such as catecholamines and histamine (18-20). In recent years, a number of important SNPs in *SLC22A3* have been identified to down-regulate *SLC22A3* mRNA expression and OCT3 protein function. Thus, these SNPs have been associated with prognosis and treatment of prostate cancer, colorectal cancer, coronary artery disease (CAD), and other human diseases (20, 28-30, 34-41). Out of many SNPs in the 5' UTR, 3'UTR, and coding regions of *SLC22A3*, rs2048327, rs1810126, and rs3088442 were chosen in our present study for the following reason: while rs2048327 is located in an intron of *SLC22A3*, rs1810126 and rs3088442 are at 3' UTR (37, 41, 42). It has been well documented that these three SNPs effectively downregulate the expression of *SLC22A3* mRNA, thus bringing about a "global" impact on the function of OCT3 in cells or patients (41).

Table I summarizes the genotyping results of these *SLC22A3* SNPS in PBMCs from a cohort of 108 MM patients. The MAFs for rs1810126, rs2048327, and rs3088442 are 30.1%, 29.6%, and 29.2%, respectively, which are comparable to the frequencies previously reported. The genotype frequencies of

Table I. *Genotypes of 3 SNPs in SLC22A3 gene in OSU11055 patients (N=108).*

Gene	SNP ID	Genotype*						Allele			*p*-Value***
		M/M	n_1	M/m	n_2	m/m	n_3	*M*	*m*	MAF	
SLC22A3	rs2048327	T/T	54	C/T	45	C/C	9	153	63	0.292	0.99
SLC22A3	rs1810126	C/C	53	C/T	45	T/T	10	151	65	0.301	0.99
SLC22A3	rs3088442	G/G	54	A/G	44	A/A	10	152	64	0.296	0.99

SNP, Single nucleotide polymorphism; SLC22A3, solute carrier family 22 member 3A; MAF, minor allele frequency. *The number of patients (n) with genotypes for major (M) and minor (m) alleles. ***Two-tailed χ^2 tests were used to analyze the differences between the actual genotype frequency and the genotype frequency predicted from Hardy-Weinberg equilibrium (HWE).

these three SNPs are in Hardy-Weinberg equilibrium (p=0.99), indicating that there is no genetic bias in our study cohort. Further linkage disequilibrium (LD) analyses show that these three SNPs are closely associated with each other (all p<0.00001). The r^2 of rs2048327/rs1810126, rs2048327/rs3088442, and rs1810126/rs3088442 are 0.96, 0.98, and 0.98, respectively; the D' values between any two of these three SNPs are close to 1.00. Such strong associations in linkage disequilibrium would be ascribed to the chromosomal proximity of these three SNPs. In regard to the association among these *SLC22A3* SNPs, we focused on rs3088442 in the following studies.

The demographic characteristics of patients in the study cohort are summarized in Table II. These 108 MM patients are evenly divided into two groups based on their rs3088442 genotypes: the group with homozygous GG genotype (wild type), and the group containing homozygous or heterozygous A variant genotype. There are no statistically significant differences between these two groups based on age, race, melphalan dose level, risk, and length of hospital stay (LOS) (p-values>0.2). Apparently, more men trended to carry rs3088442 A variant than women (59.3% *vs*. 38.8%; p=0.053) in our study cohort, nevertheless, such association remains to be further investigated in large cohorts of MM patients.

SLC22A3 rs3088442 was associated with the severity of oral mucositis in MM patients with autoHSCT-HDM therapy. It is known that melphalan is one of the substrates of OCT3 and rs3088442 GàA variation downregulates the expression of *SLC22A3* mRNA in cells (41). Hence, we wondered if there was any potential association between rs3088442 and clinical outcomes in MM patients after melphalan treatment. Our results showed that there are no statistically significant associations between rs3088442 SNP and progression-free survival (PFS) of these patients (p=0.71) nor between rs3088442 and 90-days response in these patients (p=0.48). Contrarily, there was a statistically significant association between rs3088442 and the severity of oral mucositis in these MM patients (p=0.049). As shown in Table III, univariate analyses demonstrated that rs3088442 and the area under the plasma concentration curve (AUC) of melphalan were associated with the incidence of Levels 2 and 3 oral mucositis in MM patients. While the association between AUC of melphalan and oral mucositis has been reported in previous studies (33), to our knowledge, this is the first report on the association between *SLC22A3* rs3088442 and oral mucositis. Further multivariate analyses showed that rs3088442 was associated with oral mucositis in a way independent of AUC. In comparison with patients carrying rs3088442 GG (wild type), patients carrying rs3088442 A variants (AA and AG) tended to have severer oral mucositis. The odds ratio (OR) of having severe oral mucositis between patients carrying rs3088442 A variants and patients carrying rs3088442 GG was 3.03 (95% CI=1.06-10.1, p=0.049); after adjustment with AUC, the corresponding OR was 4.00 (95% CI=1.25-14.7, p=0.027). Taken together, our results indicate that rs3088442 is associated with the toxicity, not the efficacy (90-days response and PFS) of melphalan in MM patients.

SLC22A3 rs3088442 was associated with pharmacokinetic properties of melphalan in MM patients. As transporters of many therapeutic drugs, OCTs have been shown to be involved in the disposition of these molecules in tissues, thus impacting their pharmacokinetic and pharmacodynamic properties (18-20). To pursue this, we continued to evaluate the potential associations between rs3088442 and pharmacokinetic parameters of melphalan in these patients. As shown in Table II, there is no statistically significant association between rs3088442 and AUC of melphalan in these patients (p=0.97) nor between rs3088442 and Tmax, the time to have the maximum plasma concentration of melphalan (p=0.76; data not shown). Interestingly, patients carrying rs3088442 A variants tended to have lower renal function (CrCL) than patients with rs3088442 GG wild type [89.4 (range=9.8-165.8) *vs*. 92.7 (range=5.3-196.0), ml/min; p=0.10] (Figure 1A). Accordingly, there is a difference with borderline significance between rs3088442 and the maximum plasma concentration of melphalan (C_{max})

Table II. *Demographic and clinical characteristics of patients in the current study (N=108).*

Characteristics	*SLC22A3* rs3088442 AA/AG (n=54)	*SLC22A3* rs3088442 GG (n=54)	*p*-Value**
Age (years)			
Median (range)	58.5 (40-72)	59.5 (35-71)	0.63
<65	42 (77.8%)	41 (75.9%)	0.99
≥65	12 (22.2%)	13 (24.1%)	
Gender			0.053
Female	19 (35.2%)	30 (55.6%)	
Male	35 (64.8%)	24 (44.4%)	
Race			0.43
White	50 (92.6%)	43 (79.6%)	
Other	4 (7.4%)	11 (20.4%)	
Melphalan dose*			0.23
140 mg/m^2	11 (20.4%)	7 (13.0%)	
200 mg/m^2	43 (79.6%)	47 (87.0%)	
CrCL (ml/min)			
Median (range)	89.4 (9.8-165.8)	92.7 (5.3-196.0)	0.10
CrCL <60	11 (20.4%)	6 (11.1%)	0.14
CrCL ≥60	43 (79.6%)	47 (87.0%) (1 missing)	
AUC_{inf} (mg*min/l)			
Median (range)	858 (336-1640)	909 (403-1580)	0.97
<878 (overall median)	28 (49.1%)	24 (47.3%)	0.50
≥878 (overall median)	24 (50.9%) (2 missing)	29 (47.3%) (1 missing)	
Risk			0.22
Standard risk	27 (50%)	33 (61.1%)	
Intermediate/high risk	26 (48.1%) (1 missing)	18 (33.3%) (3 missing)	
Mucositis			0.078
0	19 (35.2%)	21 (38.9%)	
1	22 (40.7%)	27 (53%)	
2	12 (22.2%)	3 (5.6%)	
3	1 (1.9%)	2 (3.7%) (1 missing)	
Response at day 90 post-transplantation***			0.63
MR/PR	21 (38.9%)	16 (29.6%)	
VGPR/CR/sCR	20 (37.0%) (13 missing)	21 (38.9%) (17 missing)	
Length of stay in hospital (days)			0.55
Median (range)	14 (10-21)	14 (11-21)	

sd, Standard deviation; CrCL, creatinine clearance; SLC22A3, solute carrier family 22 member 3A; MR, minor response; PR, partial response; VGPR, very good partial response; CR, complete response; sCR, stringent complete response. *Melphalan dose adjusted by the patient's BSA (body surface area, m^2). **All count data were analyzed using χ^2 tests or Fisher's exact tests where appropriate, and continuous data were analyzed using *U* rank sum tests. All tests were two-sided. Missing data were not included in statistical analyses. ***For 90-day response.

Table III. *Univariate and multivariate logistic regression analyses of the severity of oral mucositis in multiple myeloma patients with melphalan therapy*.*

Factor	OR	95% CI	*p*-Value
Univariate			
Gender (female *vs*. male)	2.13	[0.76, 6.25]	0.16
Dose (200 *vs*. 140 mg/m^2)	1.62	[0.40, 10.9]	0.55
Age (>65 *vs*. ≤65)	1.97	[0.62, 5.85]	0.23
CrCL (>60 *vs*. ≤60 ml/min)	0.49	[0.15, 1.70]	0.20
AUCinf (increasing by 1.0 mg*min/ml)	1.003	[1.001, 1.006]	0.0054
Risk (intermediate/high vs standard)	0.87	[0.79, 2.42]	0.79
rs2048327 (CC/CT *vs*. TT)	3.03	[1.06, 10.1]	0.049
rs1810126 (CT/TT *vs*. CC)	2.91	[1.005, 9.70]	0.060
rs3088442 (AA/AG *vs*. GG)	3.03	[1.06, 10.1]	0.049
Multivariate			
AUCinf (increasing by 1.0 mg*min/ml)	1.004	[1.001, 1.007]	0.0043
rs3088442 (AA/AG *vs*. GG)	4.00	[1.25, 14.7]	0.027

CI, Confidence interval; OR, odds ratio; CrCL, creatinine clearance; AUC, area under curve. *Mucositis grade 0/1, NO; mucositis grade 2/3, YES.

(*p*=0.07) (Figure 1B). Patients carrying rs3088442 A variants had higher C_{max} values than patients with rs3088442 GG wild type. Taken together, these results suggest that rs3088442 (or *OCT3* downregulation) may decrease the renal clearance of melphalan, resulting in higher C_{max} of melphalan in the plasma. Consequently, the elevated C_{max} of melphalan could bring about stronger "stimuli" to oral epithelial cells, leading to severer oral mucositis.

SLC22A3 was not expressed at a detectable level in PBMCs and MM cell lines. Subsequently, we evaluated the expression of *SLC22A3* mRNA in PBMCs from MM patients. Remarkably, *SLC22A3* mRNA expression was not detected in PBMCs from all 108 MM patients, regardless of their rs3088442 genotypes (data not shown). Further studies using MM cell lines with different rs3088442 genotypes showed that none of these MM cells had detectable levels of *SLC22A3* mRNA [H929, MM1S, rs3088442 GG; U266, rs3088442 AG; LP1, rs3088442 AA] (Figure 2). In comparison, *SLC22A3* mRNA was expressed in HEK293 [rs3088442 GG] and four oral cancer cell lines [SCC22A, rs3088442 GG; CAL27, rs3088442 AG; SCC4, SCC83-82, rs3088442 AA]. Additionally, no publication has been found in our recent PUBMED search for *SLC22A3* expression in blood cells. Therefore, our results indicate that *SLC22A3* is not transcriptionally expressed in PBMC and MM cancer cells. In regard to the well-known similarities in molecular characteristics between PBMC and bone marrow plasma cells (BMPCs) from the same MM patient (5), it is reasonable to anticipate that *SLC22A3* is not expressed in BMPCs. As such, OCT3 would not function in cell uptake of melphalan in BMPCs, which underlies our observation that *SLC22A3* rs308442 is not associated with 90-days response nor PFS in MM patients with melphalan therapy.

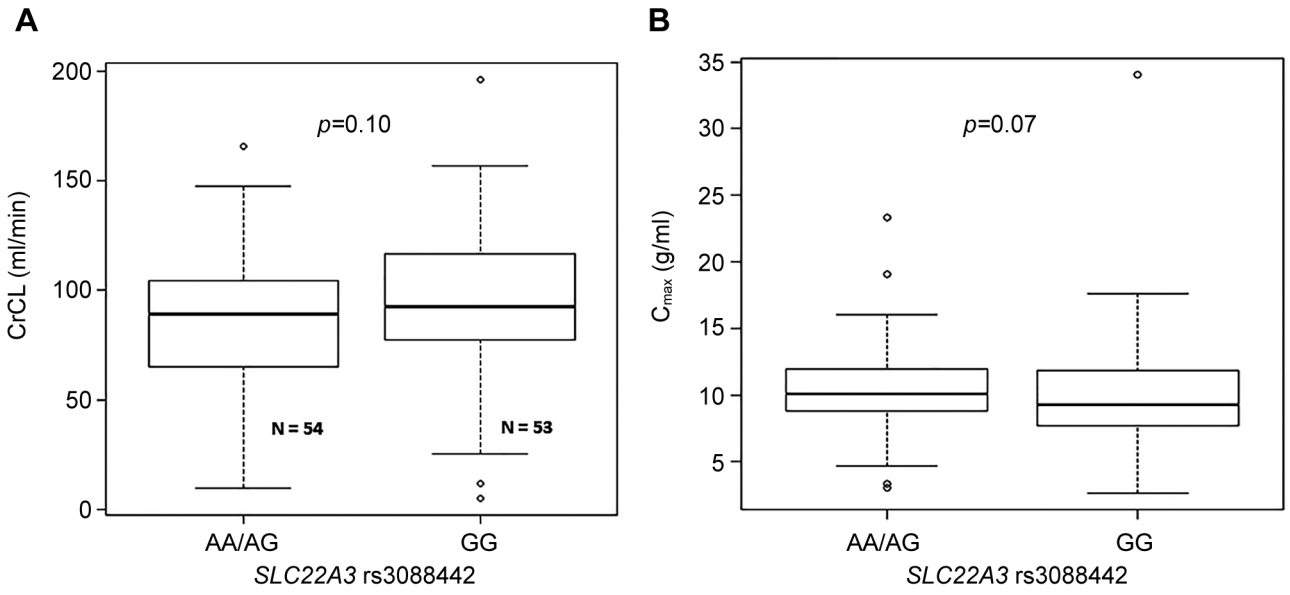

Figure 1. *Comparisons of PK parameters of melphalan between MM patients with different SLC22A3 rs3088442 genotypes. (A) CrCL; (B) C_{max}. Two-sided U rank sum tests were used to analyze the data.*

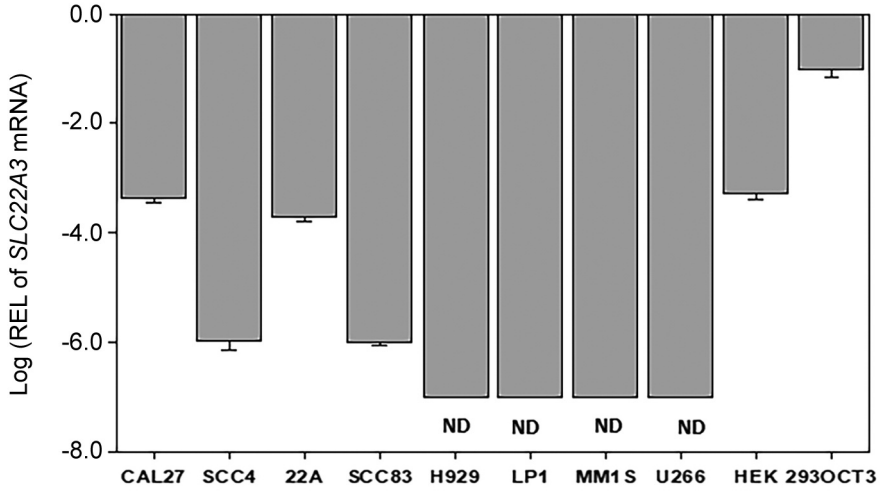

Figure 2. *mRNA expression of SLC22A3 in different cancer cell lines. RNA was extracted from PBMCs from MM patients (prior to melphalan treatment) and cDNA was synthesized as previously described. The expression level of SLC22A3 was quantitatively assessed using pre-validated Taqman® primers/probes (Hs00959834_m1 and Hs00193725_m1, respectively) on a QuantStudio™ Flex system. GAPDH was used as an endogenous reference for normalization (Taqman® assay ID: 4333764-0805024). The relative expression level (REL) of a target was determined using a comparative Cq method in which REL was defined as $2^{-\delta Cq}$. Experiments were conducted in triplicate. ND, Not detectable. HEK, HEK293; 293OCT3, HECK293_OCT3.*

SLC22A3 overexpression in HEK293 kidney cells increased its resistance to melphalan. To further investigate the potential association between *SLC22A3* expression and renal clearance of melphalan, we assessed the cytotoxicity of melphalan in HEK293 and *SLC22A3*-transfected HEK293 cells as previously described (33). While stable *SLC22A3* transfection led to an increase of about 3 fold in *SLC22A3* mRNA expression in HEK293 cells (Figure 2), the IC_{50} value of melphalan in *SLC22A3* -transfected HEK293 cells, 4.20±0.62 μM, was significantly higher than the corresponding value in

Table IV. *IC_{50} values of melphalan in oral and kidney cells*.*

Cell	rs3088442 genotype	IC_{50} (μM)
H929, multiple myeloma cell line	GG	1.30±0.12
LP1, multiple myeloma cell line	AA	>25.0
MM 1.S, multiple myeloma cell line	GG	1.21±0.42
U266, multiple myeloma cell line	AG	14.8±1.83
CAL27, oral cancer cell line	AG	4.76±0.28
SCC4, oral cancer cell line	AA	3.48±0.28
SCC22A, oral cancer cell line	GG	4.54±0.42
SCC83-82, oral precancerous cell line	AA	4.49±0.36
HEK293, kidney cell line	GG	1.03±0.02
HEK293_OCT3, transfected kidney cell line	GG	4.20±0.62

IC_{50}, the concentration of melphalan required to inhibit 50% of cell viability. *Cell viability assays were performed using a WST1 kit (Roche, Indianapolis, IN, USA). Experiments were performed in at least three replicates. IC_{50} values were determined using a four-parameter model as previously described (32).

HEK293 cells, 1.03±0.02 μM ($p<0.01$) (Table IV), indicating that *SLC22A3* overexpression in *SLC22A3*-transfected HEK293 significantly enhances its resistance to melphalan. While previous studies have demonstrated that OCT3 participates in the efflux of some drugs (such as metformin) (24, 27, 43), our results support the role of OCT3 in the efflux of melphalan in HEK293 cells. As such, elevated *SLC22A3* expression would increase the efflux of melphalan and decrease the accumulation of melphalan and its cytotoxicity in HEK293 cells. In patients with autoHSCT-HDM therapy, rs3088442 A-carriers would have downregulated *SLC22A3* expression in renal tubule cells, resulting in the decreased efflux of melphalan into the urine, *i.e.*, decreased renal clearance of melphalan, in these patients.

SLC22A3 expression did not impact the chemoresistance to melphalan in oral epithelial cells. Since oral mucositis is a "local" and tissue-specific pathological response to melphalan toxicity in MM patients (8), we further investigated the resistance to melphalan in oral cancer cell lines with different rs3088442 genotypes. As shown in Table IV, regardless of their different rs3088442 genotypes, all four tested oral cancer cell lines had comparable IC_{50} values, indicative of similar sensitivities of melphalan. As shown in Figure 2, the mRNA expression levels of *SLC22A3* were comparable between SCC4 (rs3088442 AA) and SCC83-82 (rs3088442 AA) ($p=0.83$), between CAL27 (rs3088442 AG) and SCC22A (rs3088442 GG). Moreover, SCC4 and SCC83-82 had significantly decreased levels of *SLC22A3* mRNA in comparison with CAL27 (rs3088442 AG) or SCC22A (rs3088442 GG) (p values<0.001), which is arguably consistent with the finding that rs3088442 GàA variation downregulates the expression of *SLC22A3* mRNA (41). Taken together, these results demonstrate that the chemoresistance to melphalan in oral cells is independent of rs3088442 and the expression level of *SLC22A3* mRNA. Accordingly, the observed association between rs3088442 and oral mucositis in MM patients may be ascribed to different plasma concentrations of melphalan (to be specific, C_{max}), rather than the difference in *SLC22A3* mRNA expression in oral cells/tissues in MM patients.

Discussion

OCT3 is a transmembrane transporter with many therapeutic drugs as its substrates, one of which is melphalan. It is known that OCT3 has a very broad tissue expression pattern. *SLC22A3* mRNA is highly expressed in kidney, liver, heart, and skeletal muscle, and to a lesser extent, in lung and brain; it is also expressed in neurons, glial cells, epithelial cells, and many cancer-derived cell lines, including prostate, kidney, and oral cancer cell lines (20-24). However, our results show that *SLC22A3* mRNA was expressed in kidney and oral cells, but not in MM cells nor PBMCs of MM patients, indicating that *SLC22A3* expression is not ubiquitous and OCT3 is not the major transporter mediating mephalan uptake and efflux in MM cells and PBMCs as well as other blood cells.

In cells/tissues with *SLC22A3* expression, the transporter function of OCT3 appears to be diverse in regard to drug uptake and efflux. In the small intestine, OCT3 mediates the absorption of cationic drugs and xenobiotics from the intestinal lumen (44, 45); in the heart, OCT3 appears to be the most important cationic transporter to uptake drugs (46, 47); in bronchial epithelium, OCT3 mediates the release of acetylcholine during extraneuronal cholinergic regulation (48); in salivary gland acinar cells, OCT3 contributes to both uptake and efflux of organic cations (such as metformin) in the salivary glands (27). In our current study, elected *SLC22A3* expression led to higher resistance to melphalan in *SLC22A3*-transfected HEK293 cells, suggesting that OCT3 contributes to melphalan efflux more likely than melphalan uptake in renal cells; otherwise, increased OCT3-mediated uptake of melphalan would enhance the accumulation of melphalan in HEK293 cells and sensitize them to melphalan. Presumably, since rs3088442 transcriptionally down-regulates the expression of *SLC22A3* (41), MM patients carrying rs3088442 A variant alleles would have reduced *SLC22A3* expression in renal cells in comparison with MM patients carrying rs3088442 GG wild-type alleles, resulting in reduced OCT3-mediated effulex of melphalan in renal cells (*i.e.* reduced CrCL of melphalan in patients). While the underlying molecular mechanisms remain to be elucidated, the differentiation in OCT3-mediated uptake and efflux of organic cations at different tissues/cells could be relevant to the fact that even though OCT3 is a bi-directional, facilitative diffusional transporter, its orientation in the cell membrane exhibits some

tissue/cell-specificities. It has been reported that OCT3 is localized at the basolateral membranes of renal epithelial cells and trophoblasts in the placenta, the luminal membranes of bronchial epithelial cells and small intestinal enterocytes, the sinusoidal membrane of hepatocytes (20-25, 27-30). In salivary gland acinar cells, OCT3 is located at both the basolateral and apical membranes (26).

It is also worthwhile to note that besides OCT3, multiple transmembrane tansporters may be involved in mediating cellular uptake and efflux of melphalan in patients. While down-regulation of SLC7A5, a potential transmembrane transporter of melphalan, changed the pharmacokinetic and pharmacodynamics properties of melphalan in MM patients (12, 16, 17, 49), overexpression of an efflux transporter *MDR1* (multi-drug resistance-1, also called P-glycoprotein, *ABCB1*) led to intracellular accumulation of melphalan, resulting in resistance to melphalan in MM cell lines (50). In regard to the dynamic nature of uptake/efflux, hydrolysis, and DNA intercalation of melphalan in cells, different melphalan transporters might function coordinately to influence the cellular response to melphan.

In conclusion, our study provided the first piece of evidence supporting the association between OCT3 function and oral mucositis in MM patients with autoHSCT-HDM therapy. Previous studies in our laboratory and other laboratories have shown that as a melphalan transport, *SLC7A5* (as well as its partner *SLC3A2*) was expressed in PBMCs from MM patients and different MM and non-MM cell lines, indicative of an extensive expression pattern of *SLC7A5* (16, 17, 49). In contrast, *SLC22A3* was not expressed in PBMCs and MM cell lines, suggesting that SLC7A5 plays a major role in the uptake of melphalan in blood cells (in comparison with OCT3) (17, 49, 51). As such, elevated *SLC7A5* expression would enhance melphalan uptake and sensitize cells to melphalan, thus resulting in lower chemoresistance to melphalan in cells and favored 90-days response in MM patients (49). In renal tubule epithelial cells, both SLC7A5 and OCT3 act as melphalan transporters, but maybe due to the membrane orientation of OCT3 (urine-facing), OCT3 might function primarily in the cellular efflux of melphalan and its renal excretion. Hence, *SLC22A3* SNPs, such as rs3088442, would down-regulate *SLC22A3* expression in renal cells, resulting in reduced renal exertion of melphalan and increased melphalan concentration in plasma. Consequently, increased melphalan concentration in plasma would cause severe oral mucositis in rs3088442 A variant-carriers. Apparently, our findings provided novel and valuable insights into personalized or precision medicine in MM patients with autoHSCT-HDM therapy. While a combination of cell biology, pharmacokinetics, and pharmacogentics approaches were used in our study, there are limitations. First, the cohort size of our current study is relatively small and the observed associations of *SLC22A3* rs3088442 with CrCL, C_{max} of melphalan, and oral mucositis need to be further confirmed in large patient populations. Secondly, OCT1, OCT2, and OCT3 have overlapping substrate specificities. To date, there is no report on the involvement of OCT1 and OCT2 in melphalan transport (25, 30), however, it is worthwhile to investigate the potential competition of OCT1 and OCT2 with OCT3 in melphalan uptake and efflux in MM patients. Futher studies in a large cohort of MM patients are ongoing in our laboratory.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

Authors' Contributions

DS, MAP, and CH initiated the original clinical trial. JL and MJP developed the concept and designed the current study. JL, AP, and JJ conducted the experiemnts and collected the data. JL, AP, and MJP analyzed the data and and drafted the manuscript. All Authors critically revised the manuscript and approved the final submitted version.

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