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ORIGINAL ARTICLE

Association of *CTH* variant with sinusoidal obstruction syndrome in children receiving intravenous busulfan and cyclophosphamide before hematopoietic stem cell transplantation

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Sinusoidal obstruction syndrome (SOS) is a severe complication of hematopoietic stem cell transplantation (HSCT) that can be fatal, often attributed to the conditioning regimen prior to HSCT. We evaluated the association of SOS risk with gene variants in cystathionase (CTH), an enzyme involved in glutathione synthesis, in 76 children receiving intravenous busulfan (Bu) before HSCT. Our results indicated an association with *CTHc.1364* G > T (OR$_{TT}$ = 10.6, 95% confidence interval (CI) = 2.16, 51.54) and SOS risk, which was sex dependent (female patients, OR$_{TT}$ = 21.82, 95% CI = 3.590–132.649). The interaction between *CTHc.1364 G > T* and another risk variant (*GSTA1*B*) was explored. A recessive model with the use of *GSTA1*B*B* and *CTH c.1364 TT* genotypes proved to be useful at predicting SOS occurrence, indicating the possibility of using these gene variants as markers of SOS occurrence and to further individualize preemptive treatment aimed at reducing SOS incidence.

INTRODUCTION

Sinusoidal obstruction syndrome (SOS) is characterized by the clinical features of rapid weight gain, ascites, painful hepatomegaly and jaundice.[1] The incidence of SOS in the pediatrics transplant population ranges between 7% and 27% and is higher than in adults.[2,3] The use of a busulfan (Bu) and cyclophosphamide (Cy) based myeloablative conditioning regimen is associated with SOS.[4] High Bu and high Cy metabolites concentrations often lead to increased risk of SOS.[5] Furthermore, large interindividual and intraindividual variability of Bu and Cy plasma concentrations have been observed after the same first dose administration, thought to be in part owing to metabolizing enzymes.[6]

Glutathione S-transferase alpha 1 (GSTA1) is the main metabolic enzyme involved in Bu's clearance (see Figure 1a). We have previously identified *GSTA1*B* (variant with reduced enzymatic activity) as a potential predictor of SOS, suggesting its potential for individualizing Bu treatment.[7] High Bu doses alone does not explain the occurrence of SOS[8] and *GSTA1*B*'s contribution is fairly small. As one of the main hypothesis of SOS occurrence involves the interaction between Bu and Cy causing depletion of glutathione (GSH),[9–11] we here investigated the variation in cystathionine γ-lyase (*CTH*) gene, which encodes an enzyme involved in the synthesis of GSH (Figure 1b). More specifically, this gene encodes an enzyme in the trans-sulfuration pathway that

converts cystathionine derived from methionine into cysteine, which is one of the three amino acids that make up GSH (see Figure 1b). GSH is an important antioxidant that prevents damage to cellular components caused by oxidative stress.[12] If an association with *CTH* is found, the aim is to also investigate whether it can add predictive value to *GSTA1* by performing gene–gene interaction models.

MATERIALS AND METHODS

Patients

The study included 76 pediatric patients who underwent myeloablative allogeneic hematopoietic stem cell transplantation (HSCT) with intravenous Bu- and Cy-based myeloablative conditioning regimen at CHU Sainte-Justine between May 2000 and August 2012. The Institutional Review Board approved the study and all patients/parents provided an informed consent form (IRB number: 2450). This study was fully registered in a public trial registry as part of an ongoing prospective European Blood and Marrow Transplantation multicenter study (registered at Clinical Trials.gov, NTC01257854). The demographic characteristics of the patients, details of disease and conditioning regimen are described in Table 1.

Treatment regimen

Intravenous Bu (Busulfex, Otsuka Pharmaceuticals, Tokyo, Japan) was administered as a 2 h infusion every 6 h from day − 9 to − 6. Bu first dose was based on the age of the patient and pharmacokinetic-guided dose

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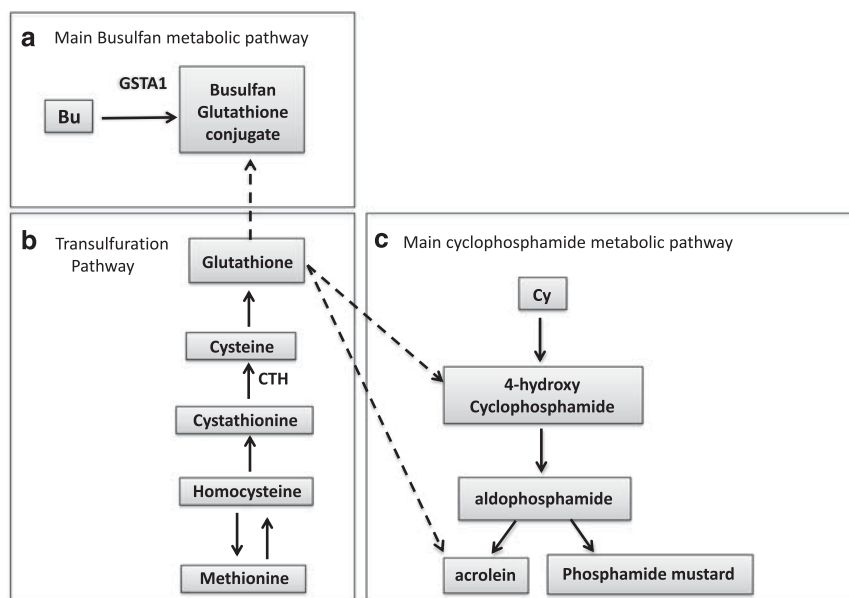E-mail: Patricia.Curtis@unige.ch

Figure 1. A simple diagram to describe the depletion of glutathione (GSH) when busulfan (Bu) is administered first before cyclophosphamide (Cy) and the role of glutathione S-transferase alpha 1 (GSTA1) and cystathionine gamma-lyase (CTH) within this hypothesis of sinosoidal obstruction syndrome (SOS) risk. (**a**) Shows the main Bu metabolic pathway, where Bu conjugates with GSH and where GSTA1 is the main catalytic enzyme. (**b**) Shows the transulfuration pathway where methionine is converted to cysteine, the main rate-limiting step for the formation of GSH. CTH is needed for the conversion of cystathionine into cysteine, which is one of the components of GSH. (**c**) Shows the main Cy metabolic pathway and its metabolites acrolein and 4-hydroxycyclophosphamide, two compounds known to be toxic to liver cells.

adjustment was performed from fifth dose onwards with a target Css of 600–900 ng ml^{-1}. Details of pharmacokinetic analysis and validation of technique is described elsewhere.[13] Acid ursodeoxycholic (Urso, Axcan Pharma, Quebec, Canada) was given for prophylaxis against SOS to 75 patients who received from 7.5 mg kg^{-1} per dose, max 500 mg (PO, BID). Urso was given the first day of conditioning till day +28 after HSCT. One patient had a second transplant with abnormal liver enzymes and received Defibrotide (Defitelio, Jazz Pharmaceuticals, Palo Alto, CA, USA) 25 mg kg^{-1} day^{-1} intravenously given in four divided doses each infused over 2 h from the first day of conditioning till day +30 after HSCT as a SOS prophylaxis. During the period of HSCT, Defibrotide was not approved in the United States and Canada but was available as an investigational drug candidate in the United States.

Definition of clinical outcome
SOS was diagnosed according to the Seattle criteria, as proposed by McDonald et al.[14] in 1984 with the day of onset extended to 50 days. The clinical course or severity of SOS was evaluated according to the description provided by McDonald et al.[15] in 1985.

Genotyping
DNA was extracted from blood collected before conditioning. CTH1364G>T rs1021737 and rs648743 single-nucleotide polymorphisms (SNPs) were chosen owing to their high minor allele frequencies (0.21 and 0.47, respectively) and potential functionality. CTH1364G>T is predicted as damaging (score = 0.04) by the SIFT protein analysis software (http://sift.jcvi.org),[16] whereas rs648743 is a promoter SNP (−1320 C>T) that could potentially abolish a glucocorticoid receptor-alpha-binding site. Furthermore, these SNPs have prior associations in pharmacogenetic studies related to homocysteine levels and stroke.[17,18] Genotyping was performed using TaqMan-based assays (C_8369524_10 and C_998383_10, respectively) on a StepOne Plus real-time PCR system under standard life technology SNP genotyping Taqman assay conditions (https://www.lifetechnologies.com).

Statistical analysis
Non-parametric (for continuous variables) and chi-square test (for categorical variables) were used to explore correlations between patient characteristics (that is, age, gender, weight, SOS prophylaxis, conditioning

regimen, Cy dose and Bu pharmacokinetic parameters) with SOS risk. Cumulative incidence of SOS in relation to the genotypes was estimated using a 1 − Kaplan–Meier curves and compared using log-rank test, in a univariate analysis. The interaction between the two gene variants was also explored, as well as the sensitivity and specificity when combined.[19] The power of the sample was calculated using G power version 3.1 (http://www.ats.ucla.edu/stat/sas/notes2) using a Goodness-of-fit test. With an expected effect size of 0.6, an alpha error probability of 0.05 and power (1 − β error probability) of 95%, this study required a total sample size of 55 patients.

Protein sequence analysis
In order to understand the effect of the mutation on CTH at the sequential level, the physiochemical properties were investigated and protein sequence analysis was performed for both wild-type and mutant form using ExPASy Proteomics Tools (www.expasy.org/tools).

Molecular docking simulation
To further understand the functionality of the CTH1364G>T variant (rs1021737 or Ser403Ile), molecular docking simulation was performed. The full-length structure of CTH is not available in the Protein Data Bank (PDB) (http://www.rcsb.org) (Available residues: 10–400, PDB ID: 3COG), thus hybrid homology modeling and ab initio approaches were used to predict the full-length model of CTH using Robetta web server (http://robetta.bakerlab.org/). Then the in silico mutant form of CTH (Ser403Ile) was generated using the COOT program.[20] Once the models were obtained, they were energy minimized by modrefiner[21] and validated by Rampage programs.[22] The energy minimized models were then subsequently subjected into protein preparation steps that included (i) addition of polar hydrogens and (ii) assignment of kollman charges and record file into the Protein Data Bank+charge+atom (PDBQT) format. The three-dimensional structure of cystathionine (CID 439258) was retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and PDB format of the substrate was obtained by the Open Babel program[23] followed by the addition of gasteiger charges, merging non-polar hydrogens, set up rotatable bonds and finally recorded into PDBQT format. The above-mentioned protein and ligand preparation steps were performed by Auto Dock Tools.[24] Auto Dock Vina was used to perform the docking calculation of substrate into both native and mutant (Ser403Ile) form of CTH.[25] For the

Table 1. Demographics of the study subjects (N = 76)

Characteristics	Patients	
	N	%
Gender		
Male	35	46.0
Female	41	54.0
Ethnicity		
Caucasian	60	79
African	11	14.5
Asian	3	3.9
American Indian	2	2.6
Diagnosis		
AML	24	31.6
ALL	6	7.9
MDS	17	22.3
Non-malignant[a]	29	38.2
HLA compatibility		
MRD	28	36.8
MUD	13	17.1
MMRD	3	4.0
MMUD	32	42.1
Stem cell source		
Bone marrow	31	40.8
Peripheral blood	1	1.3
Cord blood	44	57.9
Conditioning		
BuCy	68	89.5
BuCyVP16	7	9.2
BuCyMel	1	1.3
Serotherapy		
No	19	25
ATG	57	75
SOS prophylaxis		
Urso	70	92.1
Defibrotide	1	1.3
None	5	6.6

	Median	Range
CD34$^+$ cells ($\times 10^8$ kg^{-1})	0.007	(0.00019–1.3)
Age (years)	6.35	(0.1–19.9)
Weight (kg)	23.8	(4–95.6)
Height (cm)	122.5	(52.5–183)
BMI (kg m^{-2})	0.88	(0.54–1.45)

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globulin; BMI, body mass index; HLA, human leukocyte antigen; MDS, myelodysplastic syndrome; MMD, HLA-mismatched donor; MMRD, mismatched related donor; MMUD, mismatched unrelated donor; MRD, matched related donor; SOS, sinusoidal obstruction syndrome. [a]Non-maligant diseases included: myeloproliferative syndrome (N = 1), hemoglobinopathies (N = 10), immunodeficiency (N = 9), metabolic disease (N = 5), and hemophagocytic syndrome (N = 4).

docking calculation, the grid box size was set at 22 × 28 × 20 Å and centered on the coordinates x = 0.322, y = 0.565 and z = 0.219. Once the docking analysis was completed, the best protein–substrate complexes were selected on the basis of binding affinity and exported into the PDB format. The LigPlus[26] and PyMOL (https://www.pymol.org) programs were used for analyzing and to compare the docking results of both the native and mutant forms of CTH.
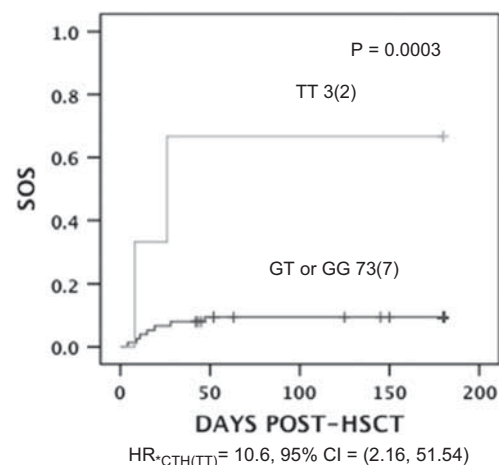
Figure 2. Association of sinosoidal obstruction syndrome (SOS) risk with *CTH c.1364G > T*. The plot shows the number of patients in each curve with and without *CTH c.1364 TT* and the number of individuals with SOS given in parenthesis. The *P*-value is shown above the curves, estimated by log-rank test for the cumulative SOS incidence between the genotype groups. Risk of SOS associated with *CTH TT* carriers in expressed as hazard ratio (HR) with 95% confidence interval (CI) indicated below the plot. HSCT, hematopoietic stem cell transplantation.

RESULTS

The overall incidence of SOS was 11.5%. Genotype frequencies for *CTH* c.1364G > T within our sample were GG (61.8%), GT (34.2%) and TT (3.9%), while for rs648743 they were TT (28.4%), CT (49.3%) and CC (22.4%). Both SNPs were in Hardy–Weinberg equilibrium and the minor allele frequency resembled Hapmap populations. No significant differences in Bu clearance and Css were observed between the *CTH* c.1364G > T or rs648743 genotype groups. There were no significant correlations between SOS risk and other features (SOS prophylaxis, conditioning regimen, weight, age, Cy dose, sex, Bu, Css).

A significant association was found between *CTH c.1364* TT genotype and SOS, (odds ratio of 10.6 (95% confidence interval (CI); 1.5–51.54) *P* = 0.003; Figure 2). As in our previous study the association between *GSTA1* and SOS incidence was mainly in female patients, we also performed the analysis following stratification by sex. In girls only, there was a relationship between SOS risk and *CTH c.1364 TT* (odds ratio of 22.40, 95% CI = 3.69, 136.17), *P* = 0.000001). In fact in this sample, the association seen with *CTH c.1364 TT* is exclusively in girls. No significant association was found between *CTH* rs648743 and SOS risk. We extended this analysis by performing a gene–gene analysis using a recessive model for both *CTH* c.1364G > T and *GSTA1*B* in female patients only. The analysis demonstrated that these two variants combined (Figures 3c and d) provided good predictability of SOS risk that was slightly better than the individual models (see Figures 3a and b) in terms of sensitivity and accuracy although they appear to be independent risk factors.

For protein sequence analysis, the main differences are observed in two posttranslational modification sites, namely, O-GalNAc (mucin type) glycosylation and serine-dependent phosphorylation. Further exploration of these two posttranslational modifications using GlycoEP[27] and IsoGlyp[28] revealed no evidence for differences in the glycocylation sites. On the other hand, for phosphorylation using DisPhos[29] it is suggested that Ser403 can be phosphorylated and this is lost with Ile403.

Docking calculation suggested that the native form (−5.0 kcal mol^{-1}, see Figure 4a) of CTH showed higher binding
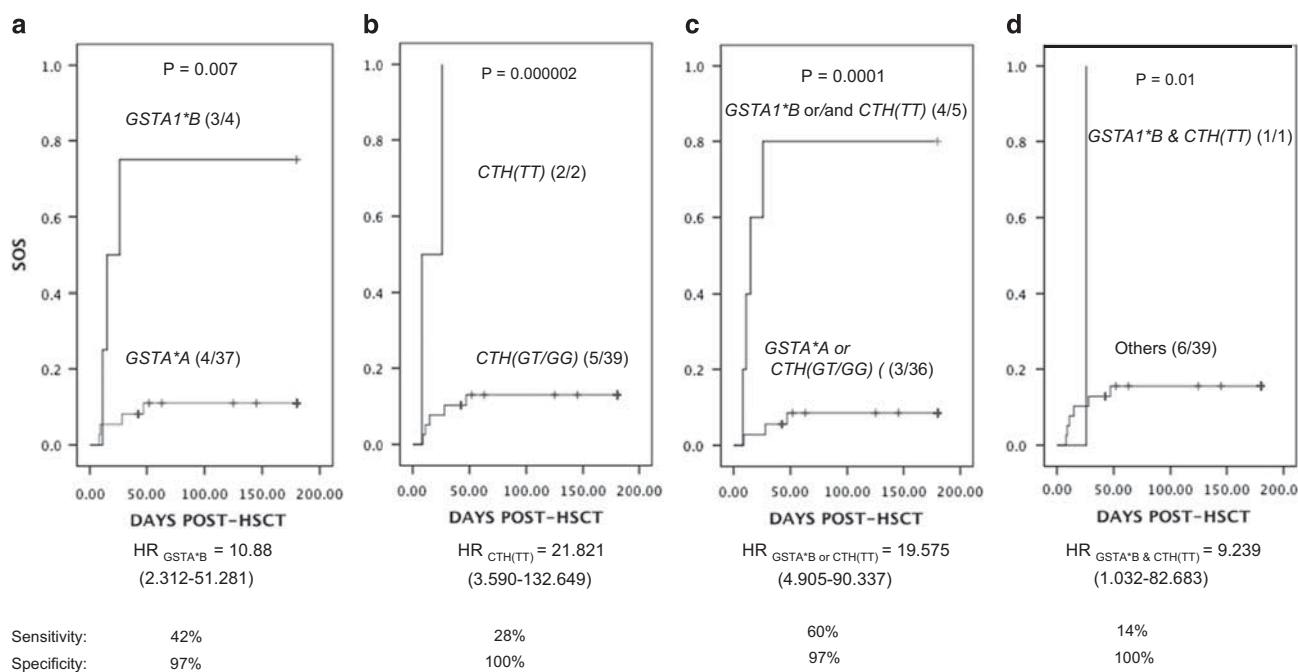
Figure 3. Association of sinosoidal obstruction syndrome (SOS) risk with different combinations of *GSTA1*B* and *CTH c.1364G>T* or alone. The plots show the number of patients in each curve with *GSTA1*B* (**a**), *CTH c.1364 TT* (**b**), *GSTA1*B* and or *CTH c.1324 TT* (**c**), GSTA1*B and CTH c.1324 TT (**d**) and the number of individuals with SOS is given in the parenthesis. The *P*-value is shown above the curves, estimated by log-rank test for cumulative SOS incidence between the genotype groups. Risk of SOS associated is expressed as hazard ratio (HR) with 95% confidenc interval (CI) indicated below the plot for each.

affinity toward the substrate in comparison to the mutant form (-4.7 kcal mol^{-1}, see Figure 4b).

DISCUSSION

The proposed mechanism by which Bu may increase the risk of SOS is GSH depletion at high doses.[30] Mice experiments suggested that activation of GSH synthesis through the increase of liver cysteine content is essential for clearance and detoxication of Bu.[11] Our previous analysis showed a tendency of higher SOS incidence with a first Bu dose Css >600 ng ml^{-1}.[7] Furthermore, we reported the association between *GSTA1*B* and SOS, where *GSTA1*B* carriers had a greater risk of SOS. In accordance with the functional effect of this variant and its impact on Bu clearance, the association is likely to be due to the reduced rate of Bu metabolism.[7] Other proposed risk factors include the depletion of GSH caused by Bu conjugation influencing elimination of Cy and its toxic metabolites (4-hydroxycyclophosphamide and acrolein, see Figure 1c).[31,32] Cy's toxic metabolites if not cleared tend to accumulate in area 3 of the hepatic acinus as this area is rich in P450 but poor in GSH, consequently causing damage to hepatocyte and sinusoidal endothelium.[33] CTH is vital for the production of cysteine, which is one of the components of GSH and also the rate-limiting amino acid in GSH biosynthesis.[34] Interestingly, an increase in plasma levels of homocysteine and cysteine are strongly correlated with severity of liver damage in children, whereas total GSH levels were significantly lower in these subjects.[35] The *CTH c.1364G>T* variant is located in exon 12 and causes a serine to isoleucine change but the functional role of this change is unknown. However, there are several studies that suggest a functionality of this variant. Two studies in adults found a significant increase of homocysteine in TT carriers compared with non-TT carriers.[17,36] Elevated homocysteine concentrations are often associated with elevated cystathionine concentrations, suggesting an increase in production or decrease in degradation possibly linking this variant to GSH

depletion. Given a conserved residue of this polymorphism, its functional effect is suggested and patients with T allele may be at a greater risk of SOS owing to lower synthesis of GSH. Exploration of posttranslational modifications with S403I points to a possible loss of two phosphorylation sites when Ile403 is present. Furthermore, the docking simulation suggests a functional change for TT genotype. Essentially, the wild type of CTH has a higher affinity (-5.0 kcal mol^{-1}) toward cystathionine compared with the mutant form (-4.7 kcal mol^{-1}). However, because the Ser403 or Ile403 C-terminus is not directly involved in the active site interaction, it can be hypothesized that it is the stability of the substrate that is decreased in the case of the mutant allele. Further experimental studies are necessary to validate these theoretical calculations.

Our study also demonstrated that the SOS risk linked to the CTH variant was limited to girls. An explanation for this association is unclear but may have something to do with the general sex differences in amino-acid levels produced by the tran-sulfuration pathway (that is, methionine, homocysteine, cystathionine, cysteine). For example, in a study comparing serum total homocysteine levels, boys were seen to have significantly higher homocysteine levels compared with girls starting as early as 10 years, suggesting higher amino-acid turnover for boys.[37] The significance of this is unclear but it does demonstrate sex difference within the pediatric population of amino-acid concentrations within this pathway, which may explain why girls might respond differently to drug toxicities.

As the clearance of Bu is mainly determined by hepatic GSTA1 activity and conjugation with GSH, which also contributes a great deal to Cy detoxification, it seems likely that cellular depletion of GSH occurs if Bu is administered first. Thus, as all but one of the patients who had occurrences of SOS in our study were given 200 mg kg^{-1} of Cy after Bu, the SOS seen in our patients could be related to Cy toxicity potentiated by Bu depletion of glutathione. This may be further exacerbated in patients who possessed either *CTH c. 1364 TT* or *GSTA1*B* variants.
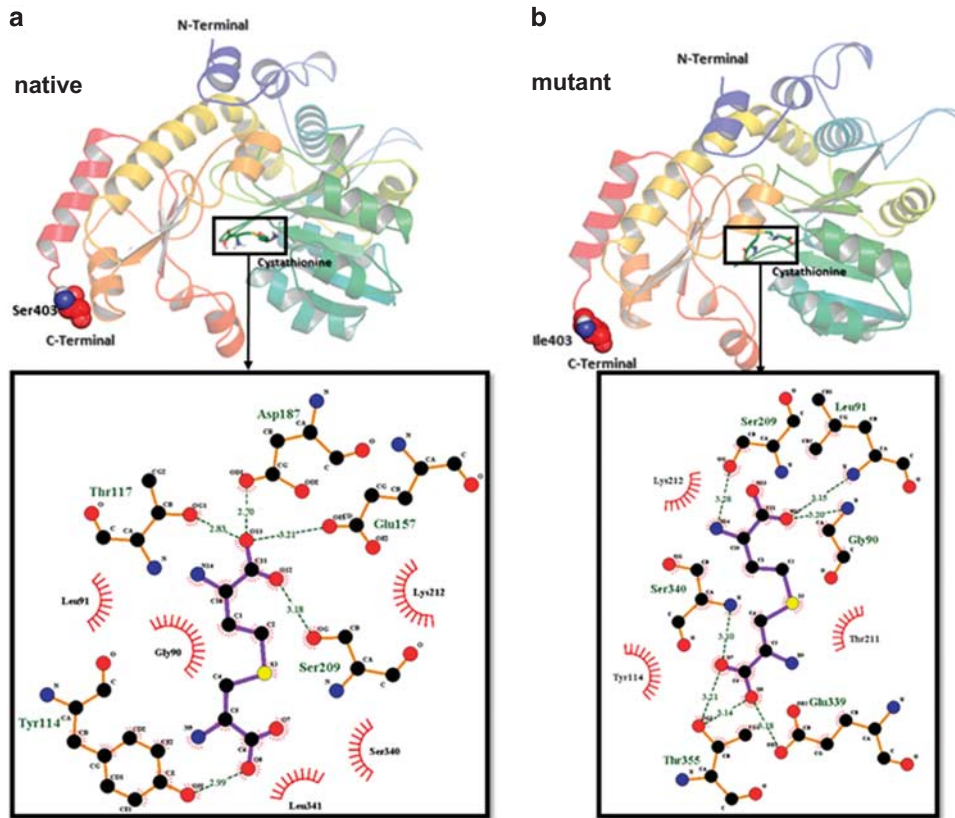
Figure 4. Binding orientation of cystathionine with cystathionine gamma-lyase (CTH). Two-dimensional representation of CTH (native (**a**) and mutant (**b**)) with substrate. The three-dimensional representation of protein–ligand complex was prepared by PyMOL. The LigPlot shows the amino-acid residues of target enzyme around the ligand molecule with hydrogen bond and hydrophobic contacts.

Table 2. SOS severity vs *CTH c.1364* G>T and *GSTA1*B* − 69 C>T variants in females only

SOS severity	CTH c.1364 G>T and GSTA1*B C>T		Total
	No risk genotype	At least one risk genotype	
Mild	2	0	2
Moderate	0	2	2
Severe	1	2	3
Total	3	4	**7**

Abbreviation: SOS, sinosoidal obstruction syndrome.

Furthermore, the gene–gene interaction analysis revealed that the presence of either risk factor or in combination might predispose individuals to developing SOS after receiving Bu followed by Cy. Thus these results show that either of the models shown in Figures 3a–d could be used to predict the occurrence of SOS, some with more sensitivity than others. The importance of genotyping both variants may be linked to the severity (see Table 2). The main limitation of this study is the small sample size as well as it being a non-homogenous disease cohort and thus the inability to perform more complex multivariate analysis. A larger pediatric study is on going in order to confirm these results.

In the early 1990s, it was shown that adding a third alkylating agent to the conditioning regimen increased the incidence of SOS significantly.[9] In the present population, 10% of the children were given three alkylating agents, either BU/CY/VP16 ($N = 7$) or BU/CY/Mel ($N = 1$). The incidence of SOS was 1 out of 8 (12.5%). This patient had neither *CTH* nor *GST* risk genotype, which might suggest that three alkylating agents increases the risk of SOS; however, it also suggests that three alkylating agents were not a confounding factor to the genetic effects.

CONCLUSION

We hypothesize that the association with SOS in this cohort involves the following combinations: (1) high first Bu dose, (2) the slow metabolism of Bu by the *GSTA1*B* variant, (3) the interaction between Bu and Cy, (4) the slow synthesis of GSH in *CTH c.1364 TT* carriers, and (5) the lower baseline GSH levels in girls. This study suggests that *GSTA1* and *CTH* gene variants may identify female patients at risk of SOS who might need a better SOS prophylaxis, for example, by administering defibrotide (Defitelio, Jazz Pharmaceuticals).[2] The administration of Cy before Bu or its replacement by Fludarabine could be also considered to avoid or reduce toxicity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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