Genotype-phenotype analysis of *CYP2C19* in the Tibetan population and its potential clinical implications in drug therapy

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Abstract. Cytochrome P450 2C19 (*CYP2C19*) is a highly polymorphic gene, it codes for a protein responsible for the metabolism of multiple clinically important therapeutic agents. However, there is currently no available data on the distribution of *CYP2C19* mutant alleles in the Tibetan population. The aim of the present study was to identify different *CYP2C19* mutant alleles and determine their frequencies, along with genotypic frequencies, in the Tibetan population. The whole *CYP2C19* gene was amplified and sequenced in 96 unrelated, healthy Tibetans from the Tibet Autonomous Region of China, the promoter region, exons, introns and the 3'-UTR were screened for genetic variants. Three novel genetic polymorphisms in *CYP2C19* were detected among a total of 27 different mutations. The allele frequencies of *CYP2C19**1A, *1B, *2A, *3A and *17 were 50, 28.13, 15.10, 5.21 and 1.56%, respectively. The most common genotype combinations were *CYP2C19**1A/*1B (56.25%) and *1A/*2A (30.21%). One novel non-synonymous mutation (Asn to Lys) in CYP2C19 was identified, and this mutation was predicted to be intolerant and benign by SIFT and PolyPhen-2, respectively. The observations of the present study may have important clinical implications for the use of medications metabolized by *CYP2C19* among Tibetans.

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Introduction

The cytochrome P450 2C (*CYP2C*) subfamily of enzymes metabolizes ~20-30% of all pharmaceuticals in use today (1). *CYP2C19* comprises 16% of the *CYP2C* subfamily (2) and is involved in the metabolism of a range of clinically important compounds, including the antiplatelet therapeutic agent clopidogrel; anticonvulsants such as phenytoin and diazepam; proton pump inhibitors such as omeprazole and lansoprazole; tricyclic antidepressants such as amitriptyline and clomipramine; and the selective serotonin reuptake inhibitor citalopram (3-7).

An association has been identified between *CYP2C19* genetic variation and therapeutic outcomes, adverse drug reactions and treatment failures (8). Among the various characterized polymorphic variants of *CYP2C19*, the most common loss of function mutations are *CYP2C19**2 (681G>A, rs4244285) and *CYP2C19**3 (636G>A, rs4986893) (9). By contrast, the common *CYP2C19**17 allele (-806CNT, rs12248560) has been associated with increased gene expression and enzyme activity (9). *CYP2C19* exhibits genetic polymorphisms among different races, as demonstrated by variations in the metabolism of therapeutic agents (10). A previous study evaluated enzymatic activity and genotypic association of *CYP2C19* among Chinese, Korean, Japanese and Caucasian populations (11).

Tibet is one of the oldest ethnic groups in China, with their own spoken and written language. To the best of our knowledge, no genotypic information on *CYP2C19* mutants in this population is currently available. The aim of the present study was to determine *CYP2C19* mutant allele and genotype frequencies in a healthy Tibetan population. The results were compared with *CYP2C19* genetic polymorphisms in other populations. The results of the present study may assist in predicting adverse effects and optimization of dosage and

Table I. Primers and amplicon sizes for cytochrome P450 2C19.

Primer	Primer sequence (5'→3')	Polymerase chain reaction product size (bp)
Promoter	F: GCCTGTTTTATGAACAGGATGA	918
Promoter	R: TAAGACAACCGTGAGCTTGC	
Exon1	F: ACAGAGTGGGCACTGGGACGA	844
Exon1	R: GGTCCTAAACCCACAGCTGCTTCC	
Exon2_3	F: TTGTCTGACCATTGCCTTGA	833
Exon2_3	R: TCTCAGCTTCAAACCCTGCT	
Exon4	F: CCCCAACTATTCTCACCCTTT	916
Exon4	R: AAAGTGTGAATTGAAGGACAAGC	
Exon5	F: TCAGGTTGTGCAAACTCTTTT	908
Exon5	R: CCTTCACTCACTTTTTGATGGA	
Exon6	F: ATGTTGGTAAGTATACAATGTGAGT	386
Exon6	R: TCACACCATTAAATTGGGACAGA	
Exon7	F: TTTTGATTGGAAATTTTAGTCCATT	921
Exon7	R: TCAGTTCTTTCCAAACTGACCT	
Exon8	F: GTCACTGGCCTTAAGCTCATGCCT	718
Exon8	R: CCCAGCCTAGGGGGTGAGGG	
Exon9	F: TGAGAGTAGGGGAGGTGAAGA	907
Exon9	R: GATGACGGGTCAGAAGAAGC	
3'-UTR	F: ACGGATTTGTGTGGGAGAGGGC	674
3'-UTR	R: AATGCTCAGCCAAAATAGCTTCCCT	

bp, base pairs; UTR, untranslated region.

treatment with medications metabolized by CYP2C19 in the Tibetan population.

Materials and methods

Participants. A group of 96 normal, healthy, non-related Tibetans (including 48 males and 48 females) were recruited between October and December 2009 from Xizang Minzu University in Xianyang. All participants were Tibetan Chinese living in the Tibet Autonomous Region of China, with a minimum of three generations of paternal ancestry within this ethnic group. Subjects with any type of medical illness, organ transplant, drug/alcohol addiction or those who were pregnant were excluded from the study. These exclusion criteria were used to minimize factors that may have influenced genetic variation in the genes of interest.

The present study was approved by The Human Research Committee of the Xizang Minzu University for Approval of Research Involving Human Subjects; all subjects were informed, verbally and in writing, about the experimental procedures, confidentiality and the purpose of the study. All participants gave their written informed consent prior to enrollment in the study.

Genotyping of CYP2C19. A blood sample (5 ml) was taken from each subject in an EDTA tube (Jiangsu Kangjie Medical Devices Co., Ltd. Jiangyan, China) and DNA was extracted using the GoldMag-Mini Whole Blood Genomic DNA Purification kit (GoldMag Co., Ltd., Hainan, China), according to the manufacturer's instructions. Primers (presented in Table I; Sangon Biotech, Shanghai, China) were designed to amplify the 5'-flanking regions, all exons, and all introns of the *CYP2C19* gene. Polymerase chain reaction (PCR) was performed for all single nucleotide polymorphisms (SNPs) in 10 μl reactions with 1 μl template DNA, 5 μl HotStar Taq Master mix (Qiagen, Germantown, MD, USA), 0.5 μl each primer (5 μM) and 3 μl deionized water. The thermal cycling conditions were as follows: Denaturation step of 15 min at 95˚C; followed by 35 cycles of denaturation at 95˚C for 30 sec, 55-64˚C for 30 sec and 72˚C for 1 min; and a final extension step at 72˚C for 3 min. PCR products were incubated with 0.5 μl shrimp alkaline phosphatase (Roche Diagnostics, Basel, Switzerland), 8 μl HotStar PCR product, and 1.5 μl deionized water (to a total volume of 10 μl), at 37˚C for 30 min, followed by heat inactivation at 80˚C for 15 min. Purified PCR products were sequenced directly using the ABI Prism BigDye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA), using an ABI Prism 3100 sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Statistical analysis. Sequencher 4.10.1 (http://www.genecodes.com/) software was used to analyze the sequences, including manual curation, fragment assembly and mutation detection. *CYP2C19* variants were designed based on the nucleotide reference sequence NG_008384.2, which is searched from the National Center for Biotechnology Information database

Table II. Positions and frequencies of cytochrome P450 2C19 genetic variants in the Tibetan study population.

No.	SNP	Position	Region	Nucleotide change	Allele	Frequency	Percentage (%)	Amino-acid effect
1	rs576566073	-844	Promoter	G>T		1/96	1.042	Not translated
2	rs12248560	-806	Promoter	C>T	*CYP2C19*17*	3/96	3.125	Not translated
3	/	-643	Promoter	C>T		1/96	1.042	Not translated
4	/	-597	Promoter	A>G		1/96	1.042	Not translated
5	rs17885098	99	Exon 1	C>T		86/96	89.583	Pro33[a]
6	/	483	Intron 1	G>A		1/96	1.042	Not translated
7	rs7918461	527	Intron 1	A>T		3/96	3.125	Not translated
8	rs4986893	17948	Exon 4	G>A	*CYP2C19*3A*	11/86	12.644	Trp212Ter
9	rs7088784	18911	Intron 4	A>G		22/96	22.917	Not translated
10	rs4244285	19154	Exon 5	G>A	*CYP2C19*2A*	39/96	40.625	Pro227[a]
11		19184	Exon 5	T>C	Novel 1	1/96	1.042	Leu237[a]
12		19280	Exon 5	C>A	Novel 2	1/96	1.042	IlE269[a]
13	rs12571421	19520	Intron 5	A>G		39/96	40.625	Not translated
14	rs557466494	58017	Intron 6	G>A		1/96	1.042	Not translated
15	rs28399513	79936	Intron 6	T>A		37/94	39.362	Not translated
16	rs374366253	79980	Intron 6	T>C		1/94	1.064	Not translated
17	rs3758580	80160	Exon 7	C>T	*CYP2C19*2A*	37/94	39.362	Val330[a]
18	rs3758581	80161	Exon 7	A>G		93/94	98.936	Ile331Val[b]
19	rs4917623	87106	Intron 7	T>C		79/96	82.292	Not translated
20	rs17886522	87313	Exon 8	A>C	*CYP2C19*3A*	12/96	12.500	Gly417[a]
21		87331	Exon 8	C>A	Novel 3	2/96	2.083	Asn423Lys[b]
22	rs17882572	87594	Intron 8	G>T		13/96	12.500	Not translated
23	rs17885052	87620	Intron 8	A>T		22/96	22.917	Not translated
24	rs12268020	89909	Intron 8	C>T		3/96	3.125	Not translated
25	/	90302	3'-UTR	C>T		1/96	1.042	Not translated
26	/	90325	3'-UTR	C>T		2/96	2.083	Not translated
27	rs185030154	90581	3'-UTR	T>C		3/96	3.125	Not translated

[a]Synonymous mutations. [b]Non-synonymous mutations. UTR, untranslated region; SNP, single nucleotide polymorphism.

(NCBI database, http://www.ncbi.nlm.nih.gov/). The *CYP* allele nomenclature is quoted from the Human Cytochrome P450 Allele Nomenclature Committee tables (http://www.cypalleles.ki.se/). The χ^2 test was used to compare allele and genotype frequencies, with descriptive analysis used to compare allele frequencies between the Tibetan and other populations (12). Haploview 4.1 (http://broad.mit.edu/mpg/haploview) was used to assess linkage disequilibrium (LD) and Hardy-Weinberg equilibrium for each genetic variant (13). Haplotypes were constructed from the selected tag SNPs and haplotype frequencies were derived for the Tibetan population.

Translational prediction. Non-synonymous SNPs in the *CYP2C19* coding regions were analyzed to predict the coded protein function. Two algorithms, Sorting Intolerant From Tolerant (SIFT; http://sift.bii.a-star.edu.sg/) and Polymorphism Phenotyping version 2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/), were used to perform the functional prediction of non-synonymous SNPs (14). Depending on the metabolic activity of *CYP2C19*, the subjects were divided into three phenotypic groups: Poor metabolizer (PM), extensive metabolizer and ultra-rapid metabolizer, based on CYP allele nomenclature (http://www.cypalleles.ki.se/) (15).

Results

Genetic variants. *CYP2C19* was sequenced in the group of 96 Tibetan participants (48 males and 48 females) and the results successfully identified a total of 27 *CYP2C19* polymorphisms in this population. Three of the polymorphisms had not previously been reported in either the National Center for Biotechnology Information database or the Human Cytochrome P450 Allele Nomenclature Committee tables (Table II), two of the novel polymorphisms were synonymous mutations within exon five and one was a non-synonymous mutation in exon eight. No duplications or deletions within the sequenced *CYP* genes were observed.

Allele and genotype frequency. A total of five *CYP2C19* alleles were detected in the Tibetan study population (Table III). The *CYP2C19*1A* allele had the highest frequency (50%), followed

Table III. Allele and genotype frequencies of *CYP2C19* in the Tibetan study population.

A, Allele frequencies

CYP2C19	Total (n=96)	Phenotype	Frequency (%)
*1A	96	Normal	50
*1B	54	Normal	28.13
*2A	29	None	15.10
*3A	10	None	5.21
*17	3	Increased	1.56

B, Genotype frequencies

CYP2C19	Total (n=96)	Phenotype	Frequency (%)
*1A/*1B	54	Normal enzyme activity	56.25
*1A/*2A	29	Reduced enzymatic activity	30.21
*1A/*3A	10	Reduced enzymatic activity	10.42
*1A/17	3	Increased enzyme activity	3.13

CYP2C19, cytochrome P450 2C19.

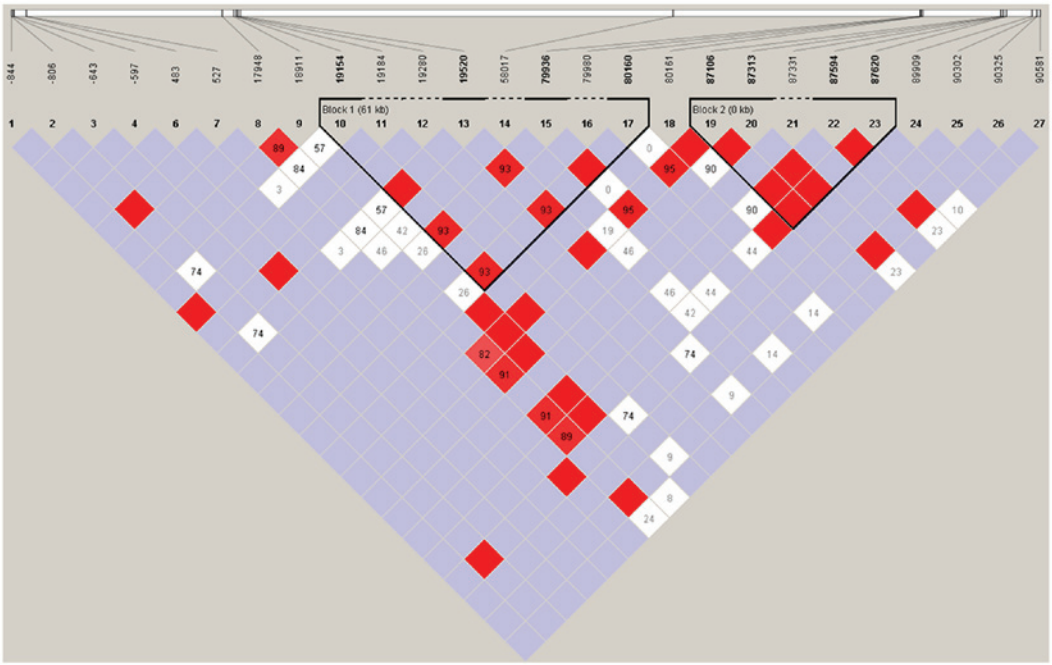

Figure 1. LD analysis of cytochrome P450, family 2, subfamily C, polypeptide 19. LD is presented with standard color schemes, bright red for very strong LD (LOD>2, D'=1), pink-red and blue for intermediate LD (LOD>2, D'<1 and LOD<2, D'=1, respectively) and white for no LD (LOD<2, D<1). Block 1 spans a 61 kb region from the nucleotide 19154 (rs4244285) in the promoter region to nucleotide 80160 (rs3758580). Block 2 includes five tightly clustered SNPs (rs4917623, rs17886522, 2C19_87331, rs17882572 and rs17885052), each with a D' value equal to 1 (indicating complete LD). LD, linkage disequilibrium, LOD, logarithm (base 10) of odds.

by the *CYP2C19**1B allele (28.13%) and the *CYP2C19**2A allele (15.10%). The other two identified alleles, *CYP2C19**3A and *17, were relatively rare with frequencies of 5.21% and 1.56%, respectively. These results indicate that alleles that do not affect the function of *CYP2C19* (*CYP2C19**1A and *1B), are the most prevalent with a combined frequency of 68.13%, followed by alleles that inactivate enzyme function (*CYP2C19**2A and *3A) with a combined frequency of 20.31%. The *CYP2C19**17 allele, which may increase the activity of *CYP2C19*, had the lowest frequency of 1.56%.

Four *CYP2C19* genotypes were identified, with frequencies ranging from 3.13 to 56.25% in the Tibetan population

Table IV. *CYP2C19* allele frequencies in different populations.

Population	n	Allele frequency (%)					Ref.
		*CYP2C19*1*	*CYP2C19*2*	*CYP2C19*3*	*CYP2C19*4*	*CYP2C19*17*	
Asian subjects							
Tibetan	96	78.13	15.10	5.21	/	1.56	Present
Chinese Han	100	67.50	25.50	2.00	0.50	3.00	(16)
Chinese Dai	193	66.30[a]	30.30[b]	3.40	/	/	(17)
Japanese	140	53.90[b]	35.00[b]	11.10	/	/	(18)
Korean	103	67.00	21.00	12.00	/	/	(19)
Vietnamese	90	62.00[a]	24.00	14.00[a]	/	/	(20)
Thai	121	59.90[b]	35.10[b]	5.00	/	/	(20)
Caucasian subjects							
Swedish	175	76.60	23.10	0.30[b]	/	/	(21)
Russian	290	88.30[a]	11.40	0.30[b]	/	/	(22)
Italian	360	88.90[b]	11.10	0.00[b]	/	/	(23)
Bolivian	778	92.10[b]	7.80[a]	0.10[b]	/	/	(24)
Faroese	312	97.10[b]	2.90[b]	0.00[b]	/	/	(25)
African subjects							
Tanzanian	251	81.50	17.90	0.60[b]	/	/	(26)
Ethiopian	114	84.00	14.00	2.00	/	/	(27)
Zimbabwean	84	86.90	13.10	0.00[a]	/	/	(28)

[a]P<0.01, compared with the data of the present study; [b]P<0.05, compared with the data of the present study. / indicates synonymous SNP mutations that have no effects on protein sequences. *CYP2C19*, cytochrome P450 2C19.

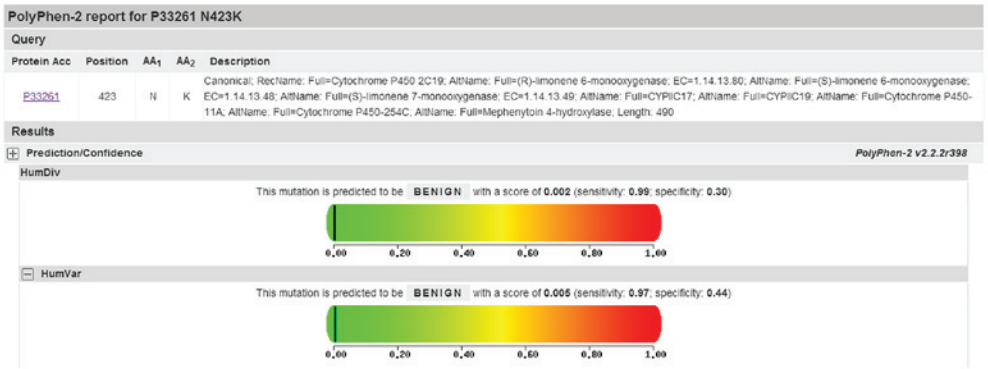

Figure 2. PolyPhen-2 prediction of functional alterations resulting from an amino acid mutation at position 423.

under study (Table III). Of the identified genotypes, one exhibits normal enzymatic activity, two exhibit reduced enzymatic activity and one exhibits increased enzymatic activity. The *CYP2C19* allele frequencies were further compared with previously published data from different countries and ethnic groups in Eastern Asia (16-19), Southern Asia (20), Europe (21-25) and Africa (26-28) (Table IV). The results of the present study demonstrated that the frequency of the wild-type allele, *CYP2C19*1*, in the Tibetan group was significantly lower than in Caucasian populations (P<0.05), however was highest in Asian populations. Furthermore, the frequencies of *CYP2C19*2* and *CYP2C19*3* were significantly higher (P<0.05) among those of Tibetan descent compared with Caucasian and African populations.

LD analysis. LD analysis was performed using Haploview with confidence intervals to define LD blocks (Fig. 1). The LD was determined for *CYP2C19* using those SNPs whose minor allele frequencies were >0.05, as SNPs with low frequency have little power to detect LD. The deviation from the expected (D') was calculated, in addition to the correlation of alleles at two loci (r^2) and the LD statistic for all possible pairs of SNPs. Two LD blocks across *CYP2C19* were identified. Block 1, the larger of the two blocks, spans a 61 kb region from nucleotide 19154 (rs4244285) in the promoter region to nucleotide 80160 (rs3758580). Block 2 includes five tightly clustered SNPs (rs4917623, rs17886522, 2C19_87331, rs17882572 and rs17885052), each with a D' value equal to 1 (indicating complete LD).

Protein function prediction. Three novel *CYP2C19* variants were identified with only one of these being a non-synonymous mutation. SIFT was used to predict the effect of mutations on CYPC219 function, with the Ala423Lys substitution predicted to affect protein function with a score of 0.01 (results are divided into four levels: Intolerant, 0.00-0.05; potentially intolerant, 0.051-0.10; borderline, 0.101-0.2; tolerant, 0.201-1.00). By contrast, PolyPhen-2 analysis predicted that this mutation is benign with a score of 0.005 (Fig. 2, the results here were divided into five levels: Benign, 0.000-0.999; borderline, 1.000-1.249; potentially damaging, 1.250-1.499; possibly damaging, 1.500-1.999; probably damaging, >2.000; and when the closer the score is to zero, the less damage is predicted).

Discussion

The polymorphic isoenzyme *CYP2C19* is responsible for the metabolism of various important therapeutic agents and *CYP2C19* polymorphisms result in inter-individual and inter-ethnic variation in the break-down of multiple therapeutic agents (29). To the best of our knowledge, the present study is the first to systematically screen a group of Tibetan individuals for *CYP2C19* variants by direct sequencing and compare these results with ethnic populations from different continents. The present study observed 27 genetic variants, including three novel polymorphisms, five alleles and four genotypes. Only one of the novel genetic variants, within exon eight, was a non-synonymous mutation.

The frequency of the wild-type *CYP2C19* allele (*CYP2C19**1) in the Tibetan study population was notably lower than in Caucasian populations, which was consistent with findings in a previous study on the Chinese Han population (30). A previous study has demonstrated that *CYP2C19**2 and *CYP2C19**3 are null alleles, which result in the total absence of enzyme activity and these two alleles account for >99% of oriental PMs and ~87% of Caucasian PMs (31). The occurrence of *CYP2C19**3 in the Tibetan subjects in the present study was significantly higher ($P<0.01$) than the frequency reported for Caucasian and African populations. These results suggest that the toxicological or pharmacological properties of medications that are metabolized by *CYP2C19* are likely to differ among Tibetans, other Asians, Caucasian and African populations. *CYP2C19**17 is within the promoter region of *CYP2C19* and this mutation is frequently reported in oriental races (16,30,32). Amongst the Tibetan study population *CYP2C19**17 had a frequency of 1.56%, which is not significantly different compared with a previous study on the Chinese Han population (30).

Various clinically important therapeutic agents are substrates for *CYP2C19,* as outlined earlier. As different mutant alleles are associated with different phenotypes and enzyme activities, *CYP2C19* genotypic and phenotypic analysis may be used to optimize dosages, improve treatment efficacy and optimize the cost effectiveness of certain treatments (33). Clopidogrel is an inactive prodrug that requires hepatic bioactivation via *CYP2C19* to exert its effects (34). This process is impaired in PMs, such as individuals with the *CYP2C19**2 allele and, consequently, the production of the active metabolite in these individuals is reduced (35). The frequency value of *CYP2C19**2 in the present Tibetan study population was between that of the other Asian groups and Caucasians, thus, it may be recommended that the dosage of clopidogrel should also lie between those used for the two populations.

An analysis of novel genetic variants in the coding region demonstrated only one non-synonymous mutation, which results in an amino acid change from asparagine to lysine. The results of the protein function analysis were determined to be intolerant and benign by SIFT and PolyPhen-2, respectively. The prediction accuracy of SIFT and PolyPhen-2 is 63 and 75%, while the false positive rate is 19 and 9%, respectively (14,36). The novel genetic variants identified in the present study should be further elucidated by other means in future studies.

In conclusion, results from the present study provide a basic profile of *CYP2C19* in the Tibetan population, and may be used to determine optimal dosage recommendations, leading to individualized medicine.

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