Mutation spectrum in Chinese patients affected by congenital sideroblastic anemia and a search for a genotype-phenotype relationship

Congenital sideroblastic anemia (CSA) is a group of diseases caused by mutations of various genes involved in heme biosynthesis, iron-sulfur cluster biogenesis or mitochondrial solute transport or metabolism.[1] In this study, we investigated the mutation spectrum in Chinese patients affected by CSAs and identified pathogenic mutations in the *ALAS2* and *SLC25A38* genes in a cohort of 31 patients.

This research was approved by the Institutional Review Board of the Peking Union Medical College Hospital and informed consent was obtained from all patients and 48 healthy controls (20 male and 28 healthy Chinese females). In the differential diagnosis of microcytic anemia, we excluded deficiencies in *MFRN1*, *STEAP3* and *DMT1*. The criteria for inclusion in this study were: i) patients with bone marrow ring sideroblasts 5% or more with no secondary cause (alcohol intake or certain drug usage); ii) patients under 40 years of age at onset; iii) patients without skin lesions or photosensitivity; iv) patients without multilineage dysplasia or thrombocytosis; v) patients without *SF3B1* mutation.

The clinical features of the 18 Chinese CSA patients are presented in Table 1. All measurements were obtained before treatment, except for the clinical data of Patients 3 (Patients 2 and 3 are siblings) and 17, which were obtained after treatment.

Using direct sequencing method, we found 15 mutations in the *ALAS2* gene of 16 CSA patients (Patients 1-16). Analysis of the *ALAS2* gene of 48 healthy subjects did not reveal any of these variations. Based on their locations relative to exon boundaries, none of these novel mutations are expected to affect the RNA splicing process.[2] None of these mutations were found in the proximal promoter and the intronic enhancer of *ALAS2* gene, thus we can also rule out any regulatory effect of the base substitutions. Patients 1, 2, 4, 5, 6, 7, 8, 11, 13 and 14 developed heart disease, diabetes and/or liver fibrosis due to massive iron overload. Patients 4, 10 and 11 required blood transfusion (before pyridoxine and iron chelation treatment), while anemia in Patient 14 was not discovered until he was admitted to hospital for hemochromatosis-related symptoms. All these patients were treated by pyridoxine and iron chelation

Table 1. Clinical features and pathogenic mutations of the Chinese patients affected by CSA.

XLSA patients (*ALAS2* mutations) ID	Gender	Onset (years)	HGB (g/L)	MCV (fL)	RET %	sFER (ng/mL)	TS %	RS %	Nucleotide change	Mutation Exon	Protein	RTP	Mean relative ALAS2 activity % (± SD)
1	M	27	81	65.8	1.30	1960	ND	29	c. 577 G>T	5	V193F	Part	45.6 ± 6.3
2	M	31	79	79.2	0.80	2890	45	15	c. 606 G>A	5	M202I	Part	54.1 ± 2.0
3*	M	28	101	83.2	ND	2882	80	ND	c. 606 G>A	5	M202I	ND	54.1 ± 2.0
4	M	23	45	72.3	1.70	1750	ND	30	c. 776 T>G	6	F259C	Comp	19.7 ± 1.1
5	M	31	97	69.5	2.20	1980	82	41	c. 800 T>C	6	F267S	Part	47.3 ± 1.0
6	M	20	68	68.0	3.10	1500	ND	51	c. 871 G>A	7	G291S	No	19.4 ± 1.0
7	M	13	62	55.2	0.04	1230	92	62	c. 897 G>C	7	K299N	No	56.5 ± 4.2
8	M	19	53	54.8	2.40	1785	58	25	c. 919 C>A	7	P307T	Comp	38.5 ± 0.6
9	M	18	72	76.0	1.40	1300	58	23	c. 946 A>G	7	K316E	Part	ND
10	M	5	54	83.1	0.93	166	91	54	c. 1070 A>T	8	D357V	ND	ND
11	M	11	39	57.1	0.06	2580	ND	44	c. 1162 A>C	8	T388P	Comp	ND
12	M	30	105	68.2	0.60	1310	ND	41	c. 1349 A>T	9	H450L	Comp	ND
13	M	36	86	59.9	0.30	1530	71	28	c. 1354 C>T	9	R452C	Part	109.3 ± 3.8
14	M	34	113	67.5	0.80	1670	56	27	c. 1634 T>A	11	L545Q	Comp	86.9 ± 0.7
15	M	33	107	72.1	0.62	1000	91	32	c. 1688 A>T	11	H563L	Comp	86.6 ± 3.5
16	M	36	67	56.0	1.20	1000	ND	37	c. 1693 G>A	11	E565K	Part	ND

CSA Patients with mutations in *SLC25A38* gene ID	Gender	Onset (years)	HGB (g/L)	MCV (fL)	RET %	sFER (ng/mL)	TS %	RS %	Mutation Nucleotide change	Protein
17	F	5	45	78.0	3.1	1500	ND	31	c. 281T>A/ c. 469 G>C	I94N/ G157R
18	M	2	36	58.8	0.4	3100	ND	23	c. 689 T>C/ c. 689 T>C	L230P

ND: not determined; Onset: onset of symptom was defined as the age when an individual acquires, develops, or first experiences CSA-related conditions or symptoms; RTP: response to pyridoxine; Part: a partial response to pyridoxine, which was defined as an improvement of anemia (hemoglobin level was increased by ≥ 10 g/L) or alleviation of symptoms after treatment; Comp: a complete response to pyridoxine, which was defined as anemia was cured or nearly cured (hemoglobin level ≥ 110 g/L) after treatment; No: no response to pyridoxine was defined as anemia was not improved (hemoglobin level was not increased or increased but ≤ 10 g/L) after treatment; SD: sensory deafness. The relative residual ALAS2 activity was determined after being normalized by the corresponding HA-ALAS2 level and expressed as a percentage of the normal enzyme. Enzyme activity was measured on 3 independent experiments.

(desferrioxamine). Most patients responded to pyridoxine treatment, especially Patients 4, 8 and 11, whose anemia was significantly improved and nearly cured (hemoglobin levels increased to 112 g/L, 128 g/L and 118 g/L, respectively, after pyridoxine treatment).

Since no significant correlation has yet been found between variations and the severity of symptoms in X-linked sideroblastic anemia (XLSA) patients, we searched for a genotype-phenotype relationship in the 16 patients carrying *ALAS2* mutations based on the orthologous residues in the crystallographic structure of *Rhodobacter capsulatus* ALAS (ALAS$_{RC}$).[3] Val193, which corresponds to Val50 in ALAS$_{RC}$, is located 3 amino acids away from the glycine binding site at Asn54. The residues corresponding to Phe259, Pro307, Asp357 and Thr388 in ALAS$_{RC}$ are Tyr116, Val164, Asp214 and Thr245, respectively. These residues are proximal to the PLP binding site or directly involved in PLP binding in ALAS$_{RC}$. Lys299 corresponds to Lys156, which was assigned to the surface of ALAS$_{RC}$ that binds to sCoA through a hydrogen bond.[3] Other novel ALAS2 mutations (*M202I, F267S, G291S, K316E, H450L, R452C, L545Q, H563L* and *E565K*) correspond to residues located far away from the PLP or substrate binding sites in ALAS$_{RC}$. In this study, a mutation close to the active site was defined as a variation corresponding to an amino acid located no more than 3 amino acids away from the substrate or PLP binding site in linear proximity along the peptide chain of ALAS$_{RC}$. The 16 XLSA patients were divided into two groups: i) Group A, patients with ALAS2 deficiency dependent on mutations close to active sites (Patients 1, 4, 6, 7, 8, 10, 11, n=7); and ii) Group B, patients with ALAS2 deficiency related to variations remote from active sites (Patients 2, 3, 5, 9, 12, 13, 14, 15, 16, n=9). Student's t-test was used to compare differences in quantitative variables between the two groups. We found that age of onset was lower in Group A (n=7; 16.86±7.58 years) than in Group B (n=9; 30.78±5.49 years) (*P*=0.001). Hemoglobin concentration was lower in Group A (n=7; 57.42±14.22 g/L) than in Group B (n=9; 91.89±16.47 g/L) (P=0.001). There was no statistically significant difference in other parameters between the two groups.

To further demonstrate that mutations close to active sites have more severe impact on the function of ALAS2 enzyme, we constructed a pET32a-HA-ALAS2 expression plasmid, which encodes N-terminally HA-tagged normal human ALAS2 protein and assessed the functional consequences *in vitro* of ten mutations identified in this study based on a previously described method.[4] It can be concluded that the mean relative residual activity of ALAS2 with mutations close to active sites (n=5; 35.9±16.3%) is significantly lower than that of ALAS2 with mutations far away from these sites (n=5; 76.8±25.7%) (*P*=0.017) (Table 1). Our hypothesis can be further demonstrated *in silico* using two bioinformatic tools (PolyPhen and SIFT). We expect our results to be helpful in further investigations of the role of PLP and substrate binding sites in normal ALAS2 function and human ALAS2 structure prediction. However, since the correlation between positons of vari-

Table 2. Clinical features of 13 genetically undefined CSA patients and relatives of the probands.

Genetically undefined CSA patients ID	Gender	ACD (years)	HGB (g/L)	MCV (fL)	RET %	sFER (ng/mL)	TS %	RS %	Clinical presentations and complications
19	M	6	27	67.6	1.23	665	104.8	64	Fatigue, drowsiness
20	M	9	61	82.3	ND	ND	ND	17	Fatigue
21	M	17	23	49.7	0.23	1161.9	89	38	Fatigue, liver fibrosis, heart disease
22	M	19	74	96.1	1.62	ND	64	22	Fatigue
23	M	36	35	78.5	1.19	1151	96	52	Fatigue
24	M	40	72	100.4	ND	ND	ND	25	Fatigue
25	F	8	39	85.5	0.08	1100	68	7	Hepatosplenomegaly, dark skin, fatigue
26	F	14	60	92.6	1.73	ND	ND	44	Fatigue
27	F	16	92	83.8	ND	ND	ND	26	Myopathy, growth retardation, fatigue
28	F	21	73	58.9	0.65	ND	ND	33	Fatigue
29	F	23	88	96.7	ND	1500	92	50	Heart disease, arthritis, fatigue
30	F	34	79	89.2	1.80	ND	ND	28	Fatigue
31	F	38	67	71.3	ND	ND	ND	19	Fatigue

Relatives of the patients ID	Gender	HGB (g/L)	MCV (fL)	RET %	sFER (ng/mL)	TS %	RS %	Mutation
8A*	M	158	95.7	ND	ND	ND	ND	None
8B*	F	123	89.5	ND	ND	ND	ND	P307T/wt
11A*	M	136	93.9	ND	ND	ND	ND	None
11B*	F	112	81.2	ND	ND	ND	ND	T388P/wt

*ACD: age clinically detected; HGB: hemoglobin; MCV: mean corpuscular volume; RET: circulating reticulocytes; sFER: serum ferritin; TS: transferrin saturation; RS: ring sideroblasts in bone marrow smear; ND: not determined; wt: wild type. *8A and 8B are parents of patient 8; 11A and 11B are parents of patient 11.*

ants and severity of anemia encompasses a large overlap, our conclusion can not be used for direct interpretation and management of individual clinical cases.

Mutations in the *SLC25A38* gene have not previously been described in East Asian CSA patients. In this study, we found three novel missense mutations in the SLC25A38 gene of 2 patients (Patients 17 and 18) who had had a severe, transfusion-dependent microcytic anemia since their first decade of life. They were refractory to pyridoxine and iron chelation and became severely iron overloaded after transfusions. Of the mutated residues, Ile94 and Leu230 are highly conserved among the SLC25A38 proteins from five mammalian species (*Homo sapiens, Pan troglodytes, Macaca mulatta, Rattus norvegicus* and *Mus musculus*). Gly157 is only conserved among the SLC25A38 proteins from higher mammals, while in rodents it is replaced by serine, suggesting that this residue may not be as essential as the other two mutated SLC25A38 residues. We speculate that this may explain why Patient 17, who is compound heterozygous for I94N/G157R, displayed a later onset of symptoms than Patient 18 (Table 1), as well as other previously described CSA patients with *SLC25A38* gene mutations whose onset of symptoms ranges from birth to three years of age.[5]

Furthermore, we failed to detect any mutation in *ALAS2, SLC25A38, SLC19A2, GLRX5, ABCB7, PUS1* or *YARS2* gene of the other 13 patients (Patients 19-31), and none of them has the clinical presentation of Pearson marrow-pancreas syndrome. The pattern of inheritance of the genetically undefined diseases seems to be autosomal recessive since none of the patients has a family history of anemia. The major clinical features of these patients and some relatives of the probands are presented in Table 2.

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