Genetic basis of persistent red blood cell microcytosis in the Chinese unexplained by phenotypical testing

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ABSTRACT

Aims Hypochromic microcytic anaemia is the hallmark phenotype of thalassaemia. Current phenotypical tests do not provide a diagnosis in a small proportion of patients with red blood cell microcytosis. We aim to evaluate the genetic basis of red cell microcytosis in these cases in our Chinese population.

Methods We identified from a large cohort of 1684 unselected requests for thalassaemia testing 23 Chinese subjects who had unexplained microcytosis after phenotypical iron and haemoglobin studies. In 18 of these subjects with available DNA, extensive genotypical analysis of the α and β globin gene cluster was performed, including gap-PCR, multiplex amplification-refractory mutation system, Sanger sequencing and multiplex ligation-dependent probe amplification.

Results Occult single and double α globin gene (*HBA1, HBA2*) deletions and α thalassaemic haemoglobinopathies (Haemoglobin Quong Sze, Haemoglobin Constant Spring) were the genetic basis for the microcytosis. Occult β globin gene (*HBB*) mutations and δ globin gene (*HBD*) abnormalities masking β thalassaemia are not seen.

Conclusions A cost-effective genotyping approach for the detection of these occult globin gene mutations can be proposed. The identification of these mutations is important for making a diagnosis and for the provision of accurate genetic counselling. (This paper adds to our understanding of the genetic basis of red blood cell microcytosis in clinical practice, and it provides a cost-effective approach for genotyping in diagnostic laboratories).

INTRODUCTION

A low mean corpuscular volume (MCV) is commonly used to screen clinical requests for thalassaemia studies after exclusion of iron deficiency. Phenotypical diagnosis of β thalassaemia is typically made by measurement of the percentage of haemoglobin (Hb) A_2 by high performance liquid chromatography (HPLC) or capillary electrophoresis. In Southern Chinese and South-East Asian (SEA) populations where large deletions involving both α globin genes (*HBA1, HBA2*), such as the α SEA deletion, are the predominant causes of α thalassaemia, detection of Hb H inclusion bodies in red blood cells by supravital staining remains as the mainstay test for phenotypical diagnosis of α thalassaemia. With technical experience it is very sensitive and specific in detecting α-zero thalassaemia trait and probably also homozygous α-plus thalassaemia trait. In a small proportion of subjects with red blood cell microcytosis, these phenotypical tests yield negative results. Globin gene mutations that may show such clinical presentation fall into several groups: (1) occult α thalassaemia due to single *HBA* deletions and ultraunstable variants; (2) normal Hb A_2 β thalassaemia due to mild *HBB* mutations, large deletions of the β globin gene cluster and mutations of the β-locus control region (LCR); (3) masked β thalassaemia due to coexisting δ thalassaemia and variants; (4) amplification of *HBA* leading to α and β globin chain imbalance. In this study, we investigated the molecular basis of microcytosis in subjects who had a normal Hb phenotype. We aimed to document the occurrence and relative prevalence of various occult globin gene mutations and to establish a cost-effective laboratory approach for their detection.

METHODS

Study subjects

We screened all the 1684 records of thalassaemia studies performed in the Haematology Laboratory, Queen Mary Hospital, Hong Kong from January 2009 to December 2010. All subjects had microcytosis (MCV <82 fL) except for <1% of cases that were investigated for a suspected Hb variant. Subjects who were diagnosed as α and β thalassaemia by phenotypical testing and subjects with iron deficiency as evidenced by a low serum iron level and/or a low transferrin saturation percentage were excluded. Subjects who had documented normalisation of MCV on follow-up complete blood count analysis were also excluded. Twenty-three subjects had persistent unexplained microcytosis. Peripheral blood film examination of red blood cells showed microcytosis only. None of these cases showed prominent basophilic stippling, including the three Hb Constant Spring (CS) cases. They had a normal Hb phenotype including an unremarkable HPLC chromatogram. Eighteen of them had archive specimens available for comprehensive genotypical analysis and were recruited into this study. Patient particulars and Hb phenotypes were summarised in table 1. All 18 subjects either had a normal Hb level or mild anaemia except for three cases, who had significant autoimmune haemolytic anaemia complicating their systemic lupus erythematosus (Case 4 and 14) and juvenile inflammatory arthritis (Case 17). These complicating diseases, however, could not explain the microcytosis.

Standard α globin genotyping

Frozen whole blood samples in EDTA were retrieved for molecular studies. Genomic DNA was extracted by the standard phenol/chloroform method. Single *HBA* deletions of the 3.7 kb and 4.2 kb type and α SEA deletion were screened by a

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Table 1 Personal particulars, phenotype and globin genotype of 18 subjects with phenotypically unexplained microcytosis

Sample number	Sex/Age	RBC ($\times 10^{12}$/L)	Hb (g/dL)	MCV (fL)	MCH (pg)	Hb A_2 (%)	Hb F (%)	Hb H granules	α gap-PCR	α ARMS	α sequencing	α MLPA	β sequencing	β MLPA	δ sequencing
1	F/28	4.51	12.0	79.6	26.6	3.2	0.7	Neg	4.2 Hetero	Neg	ND	ND	N	ND	N
2	M/12	5.28	13.0	73.9	24.6	2.7	0.3	Neg	Neg	Hb CS	ND	ND	N	ND	N
3	F/39	4.89	12.0	73.1	24.5	3.0	0.3	Neg	Neg	Hb QS	ND	ND	N	ND	N
4	F/44	2.99	7.1	72.6	23.7	2.7	0.5	Neg	SEA Hetero	Neg	ND	ND	N	ND	N
5	F/42	5.09	12.0	70.9	23.6	2.5	0.4	Neg	Neg	Hb QS	ND	ND	N	ND	N
6	M/12	4.81	11.7	73.1	24.3	2.7	0.3	Neg	Neg	Neg	N	Neg	N	Neg	N
7	F/44	5.10	13.1	77.8	25.6	3.0	0.5	Neg	Neg	Hb QS	ND	ND	N	ND	N
8	F/20	4.56	11.7	78.7	25.7	2.7	0.2	Neg	Neg	Neg	N	Neg	N	Neg	N
9	M/23	5.49	14.5	78.1	26.5	2.6	0.2	Neg	Neg	Hb CS	ND	ND	N	ND	N
10	F/38	5.00	13.2	78.7	26.4	2.8	0.3	Neg	3.7 Hetero	Neg	ND	ND	N	ND	N
11	F/82	4.15	10.5	78.8	25.2	2.3	0.5	Neg	SEA Hetero	Neg	ND	ND	N	ND	N
12	M/81	5.26	13.2	79.3	25.1	3.4	0.2	Neg	Neg	Hb QS	ND	ND	N	ND	N
13	F/51	4.48	10.3	72.2	23.1	2.2	0.1	Neg	3.7 Hetero	Neg	ND	ND	N	ND	N
14	M/17	4.60	9.8	67.6	21.3	2.0	0.1	Neg	4.2 Hetero	Neg	ND	ND	N	ND	N
15	M/13	4.91	12.7	77.0	25.9	2.6	0.5	Neg	Neg	Hb CS	ND	ND	N	ND	N
16	M/8	5.56	12.8	70.5	23.0	2.9	0.3	Neg	Neg	Hb QS	ND	ND	N	ND	N
17	F/4	3.83	8.3	73.1	21.6	2.9	3.1	Neg	Neg	Neg	N	Neg	N	Neg	N
18	M/22	5.22	13.4	77.7	25.6	2.9	0.3	Neg	4.2 Hetero	Neg	ND	ND	N	ND	N

3.7, α 3.7 kb deletion; 4.2, α 4.2 kb deletion.
ARMS, amplification refractory mutation system; CS, Constant Spring; Hb, haemoglobin; Hetero, heterozygous; MCH, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; MLPA, multiplex ligation-dependent probe amplification; N, normal; ND, not done; Neg, negative; QS, Quong Sze; RBC, red blood cell count; SEA, α South-East Asian.

multiplex α gap-PCR system. Selected α thalassaemic structural variants including Hb Quong Sze (QS), Hb CS and codon 30 deletion were screened by amplification refractory mutation system. Samples negative in both tests were subjected to automated Sanger sequencing of *HBA1* and *HBA2*. All three tests were performed as previously described.[1]

Standard β and δ globin genotyping

Point mutations causing β and δ thalassaemia and structural variants were detected by automated Sanger sequencing of *HBB* and *HBD*. Procedures for both genes were the same except for the specific primers used (β PCR primers: forward (833) 5′- TCCTAAGCCAGTGCCAGAAG-3′; reverse (834) 5′-GACCTCCCACATTCCCTTTT- 3′; δ PCR primers: forward (BE2) 5′-AAGTTAAGGGAATAGTGGAATGAAG-3′; reverse (BE9) 5′-CCCATTAATGCCTTGTACGG-3′). Genomic DNA samples were amplified by an ABI 9700 thermal cycler (Applied Biosystems, Foster City, California, USA) using the following cycling parameters: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C denaturing for 40 s, 57°C annealing for 40 s, and 72°C extension for 3 min. The presequencing cycle programme was 37°C for 30 min, 80°C for 15 min. Sequencing primers for *HBD* were the same as the PCR primers. For *HBB* sequencing, apart from the forward (833) and reverse (834) primers, an additional forward primer (CA) 5′-CAATGTATCATGCCTCTTTGCACC-3′ was also used to sequence the exon 3 and the 3′ untranslated region. The sequencing cycle was started with initial denaturation at 96°C for 10 min, followed by 24 cycles of 96°C denaturing for 10 s, 50°C annealing for 5 s and 60°C extension for 4 min. Sequences were read by an ABI 3130XL sequencer (Applied Biosystems).

Detection of amplifications and uncommon deletions in α and β globin gene cluster

Multiplex ligation-dependent probe amplification (MLPA) was employed in selected cases to screen for gene dosage changes, and deletions disrupting the upstream regulatory region HS-40 and β-LCR in the α and β globin gene clusters, respectively. Probes and reaction mixture for ligation and PCR were purchased from MRC-Holland (SALSA MLPA kit P102 HBB and P140-B3 HBA, MRC-Holland, Amsterdam, the Netherlands). Testing and data analysis procedures were done as previously described.[2]

RESULTS

A summary of the globin genotyping results is shown in table 1. Of the 18 subjects tested, 15 (83%) showed an α globin gene cluster mutation, with seven samples (39%) of the deletional types (two cases of SEA type, two cases of 3.7 kb and three cases of 4.2 kb deletion) and eight samples (44%) of the non-deletional types (three cases of Hb CS, five cases of Hb QS). The remaining three samples (17%) showed normal molecular findings, with no evidence of point mutation in α and β globin genes or amplification or deletion of the α and β globin gene clusters. None of the 18 samples harboured β or δ globin gene mutations as assessed by Sanger sequencing of the promoter, all exons and the 3′ untranslated region.

DISCUSSION

It is well recognised that single *HBA* deletions and certain α thalassaemic variants such as Hb CS may present with a normal MCV and thus be missed by most thalassaemia screening programmes that are based on detection of microcytosis.[3] Even when these mutations present with a low MCV, they are not effectively identified by routine phenotypical Hb studies. Supravital staining is notoriously insensitive in picking up single *HBA* deletions, Hb CS and Hb QS because of the modest chain imbalance compared with double *HBA* deletions such as the α SEA deletion.[4] An immunochromatographic strip test which detects Hb Bart has improved detection rate for single *HBA* deletions but its sensitivity is still only 83%.[5] A small amount of Hb CS may be evident in HPLC, capillary electrophoresis and alkaline Hb electrophoresis but the detection is unreliable. The ultraunstable Hb QS chain cannot be detected by routine methods at all. These occult *HBA* mutations can result in thalassaemic syndromes with significant morbidities when they occur in a homozygous state or in compound heterozygosity with other *HBA* mutations. These include deletional and non-deletional Hb H disease which can present as Hb H hydrops foetalis in extreme cases.[6 7] The same consideration also applies to masked *HBB* mutations. Normal Hb A_2 β thalassaemia may be due to mild mutations in the 5′ untranslated region of *HBB* that have no clinical significance. However, severe β^0 mutations can also be masked phenotypically by coexisting δ thalassaemic mutations in *cis*, a large deletion involving all the genes in the β globin gene cluster or a deletion of the β-LCR. Genotypical analysis is necessary to effectively diagnose all these masked α and β and thalassaemia mutations.

Our study results show that persistent microcytosis unexplained by phenotypical testing is an uncommon finding in clinical practice. Among 18 such cases in this study, α thalassaemic mutations accounted for all 15 cases of masked globin gene mutations detected. None of the 18 cases harbour any *HBD* or *HBB* mutations. Of note, two cases with α SEA deletion did not have detectable Hb H granules. α globin genotyping was not routinely performed for cases already diagnosed by phenotypical testing to allow for direct calculation of diagnostic sensitivity of supravital staining in this study. However, from a previous study on the local Chinese population with red blood cell microcytosis, which showed a prevalence of 54% of α SEA deletion,[8] the sensitivity of supravital staining for α SEA deletion in our cohort is around 99%. This is highly acceptable for clinical diagnostic use. However, this test is observer-dependent. Use of more sophisticated phenotypical testing methods is required to diagnose α^0 thalassaemia if 100% sensitivity is aimed at.[5]

The detection of three cases of Hb CS without *HBA* deletion in our patient group is an interesting finding. It has been proposed that oxidised Hb CS binds to red blood cell membrane. This leads to membrane damage and overhydration of red blood cells, and causes normalisation of MCV despite the otherwise thalassaemic property of this variant.[9] A low MCV and mean corpuscular haemoglobin concentration associated with Hb CS is regarded as an indicator of coexisting single *HBA* deletion.[10] The finding of microcytosis in our three cases of isolated Hb CS, including one case with a very low mean corpuscular haemoglobin concentration of 24.6 pg (Case 2), does not support this claim. Basophilic stippling, as reported previously in homozygous Hb CS,[11] is absent in our cases. This is likely due to the fact that our three cases are only heterozygous. Homozygous Hb CS almost always has a normal MCV due to significant membrane damage.[11]

The cause of persistent microcytosis in the remaining five cases is uncertain. Uncommon large deletions or amplification of the globin gene clusters or disruption of their 5′ regulatory regions were not detected. Whether these cases still represent thalassaemia due to trans-acting factors outside of the globin gene clusters can be further investigated by globin chain synthesis or quantitative reverse transcriptase-PCR to document

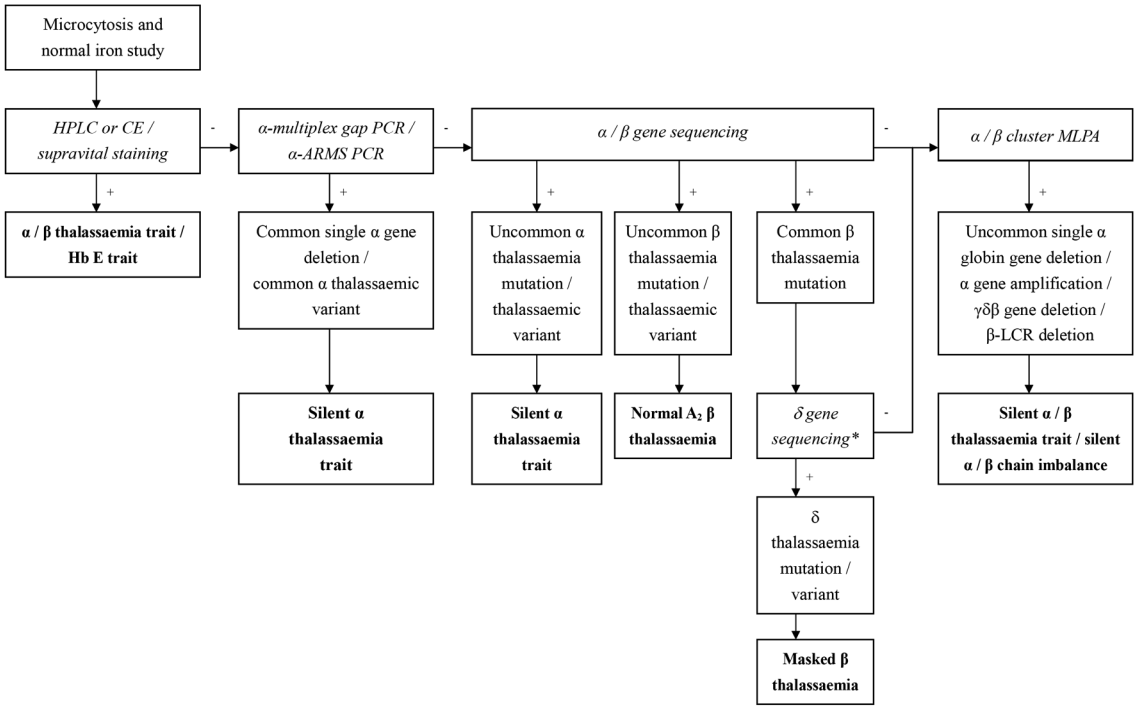

Figure 1 A proposed diagnostic algorithm for thalassaemia in Chinese patients with red blood cell microcytosis. ARMS, amplification refractory mutation system; CE, capillary electrophoresis; Hb, haemoglobin; HPLC, high performance liquid chromatography; LCR, locus control region; MLPA, multiplex ligation-dependent probe amplification. *δ Gene sequencing indicated upfront if HPLC/CE shows an abnormal peak suspicious of a Hb A_2 variant.

relative globin gene expression. Unfortunately, fresh blood samples were not available for such analysis. Atypical microcytic anaemias due to rare inherited defects of iron metabolism present either with iron overload or deficiency and this should be detectable in the serum iron profile. There may also be associated neurological and endocrine disorders, which were not present in our subjects.[12] Anaemia of chronic disease usually presents as normochromic normocytic anaemia but sometimes microcytosis can be seen as a result of iron-restricted erythropoiesis. This is reflected by low serum iron and transferrin levels and a raised ferritin level. Chronic lead poisoning is another acquired cause of microcytosis, where basophilic stippling is observed in red blood cells.

From our findings we propose a cost-effective genotyping approach to detect masked thalassaemic mutations for definitive diagnosis and genetic counselling in subjects with unexplained microcytosis (figure 1). Using simple α multiplex gap-PCR and amplification refractory mutation system, the underlying genetic cause for the microcytosis can be determined in most cases. More sophisticated and expensive molecular techniques such as Sanger sequencing and MLPA of the globin gene clusters are indicated only when the first line molecular tests yield negative results.

Take home messages

- Phenotypically unexplained red blood cell microcytosis is an uncommon clinical finding, yet a thorough search for masked thalassaemic mutations is important.
- Single *HBA* deletions and α thalassaemic structural variants account for most of these masked globin gene mutations.
- A cost-effective genotyping approach can be set up for the detection of such masked mutations.

Contributors C-CS designed the study, analysed the data, wrote the manuscript and is responsible for the overall content; AKL identified study samples, performed the experiments and reviewed the manuscript; MHT, DYN, K-SL and AYC performed the experiments and reviewed the manuscript.

Competing interests None.

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