Molecular Genotyping of Silent Beta Thalassaemia Carriers among the Healthy Adult Population in Yangon

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Beta thalassaemia trait (minor) or heterozygous carrier type has one beta globin gene defect that may be asymptomatic or cause slight anemia. Molecular genetic testing of the gene encoding the hemoglobin subunit beta (HBB) represents advanced method for detection of pre-symptomatic or silent beta thalassaemia carrier in at-risk family members and for prenatal diagnosis. Out of 516 healthy adults (age 16-45 years) living in Yangon, 103 have anemia (Hb <12g%). Twenty-one (4%) of beta thalassaemia carriers were detected by osmotic fragility test (OFT), HbF% and HbA 2% estimations were done by high performance liquid chromatography (HPLC) and hemoglobinopathies were observed by iso-electric focusing gel electrophoresis (IEF). HBB gene mutation was detected in 52 adults (10%) by using polymerase chain reaction with single strand conformation polymorphism (PCR-SSCP). The most common HBB gene mutation was CD26G>A (HbE) (66.7%). The other common five genetic mutations were CD35C>A, CD6A>T (HbS), CD71/72+A, CD17A>T and IVSII 654C>T that were diagnosed as silent beta thalassaemia carriers. Molecular screening for HBB mutation is a sensitive test and easy technique to detect silent beta thalassaemia carriers among healthy adult population after testing of essential red blood cells parameters (Hb%, MCV, MCH, RDW) by automatic blood analyzer and morphology of red blood cell by blood film examination. Screening of thalassaemia carriers and providing the proper health education in adult population are part of main strategies for prevention and control of new cases of thalassaemia major in Myanmar.

Keywords: Molecular genotyping, Thalassaemia, Adult population, HBB gene

INTRODUCTION

The thalassaemias are congenital anaemias that represent a heterogenous group of inherited red cell disorders having in deficient synthesis of one or more globin subunits of the normal human hemoglobins. There are many different types of thalassaemia such as α, β, δ or αβ depending on abnormal synthesis of globin chain that is responsible for ineffective erythropoiesis and peripheral destruction of red blood cells. Thalassaemia is one of the most common genetic disorders in the world. One-hundred and fifty million people (3% of the world's population) carry beta thalassaemia genes.[1] The incidence of β thalassaemia trait among the Myanmar was 0.54% in anemia research project.[2] Overall incidence of thalassaemia among Myanmar population is 4.3% and HbE thalassaemia is 0.04% in the previous study.[3, 4] In Myanmar, the commonest types of thalassaemia in admitted patients are beta thalassaemia major related to gene interaction between hemoglobin E trait and beta thalassaemia

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trait.[5] The prevalence of β thalassaemia in different ethnic groups of Myanmar are 1.5% in Chin, 1.0% in Rakhine and 2.5% in Bamar, 2.6% in Kachin and 6.9% in Kayin, respectively.[6, 7] Thalassaemia is mainly caused by globin gene mutation and beta thalassaemia affects one or both of the beta-globin genes. The genes controlling beta chain production are located on chromosome 11 which is a gene for beta globin components of hemoglobin. HbE is a beta chain variant in which lysine is substituted for glutamic acid in position 26. HbE is one of the unstable hemoglobin characterized by production of abnormal globin chains.[1]

In silent beta thalassaemia minor/beta + or trait/heterozygous carrier type has one beta thalassaemia globin gene defect and beta thalassaemia major/beta -0/homozygous has a complete absence of beta-globin gene. Haematological diagnosis of thalassaemia is significantly identified by morphology of red blood cells in blood film examination, osmotic fragility test and hemoglobin analysis by high performance liquid chromatography (HPLC) and isoelectric focusing electrophoresis (IEF) methods. Silent beta thalassaemia carriers have clinically normal red cell indices. Molecular detection of thalassaemic gene analysis including genotypes and genetic polymorphisms are important for diagnostic and therapeutic application for thalassaemic patients.[8] The aim of this study is molecular genotyping of silent beta thalassaemia carriers among the healthy adult population in Yangon.

MATERIALS AND METHODS

Type of study and study population

A laboratory-based descriptive and analytical study was done on a total of 516 apparently healthy adults of both sexes aged between 16-45 years who were recruited from Nursing University of Yangon in 2014. Genetic origin, outcomes and prevention of beta thalassaemia disease were explained to nursing students before taking the sample. After being approved by the Institutional Ethical Review Committee, Department of Medical Research (Lower Myanmar), informed written consents were obtained from these subjects. The drug history, family history and medical history of these subjects were recorded according to the proforma in this study. Three milliliters of blood from ante-cubital vein were collected into ethylenediamine tetraacetic acid (EDTA) blood collecting tube and stored at 4ºC until the analysis.

Laboratory procedure

All fresh whole blood samples were tested for haematological parameters by Automatic Haematological Analyzer (Pantra 60) and blood film examination. Hypochromic microcytic anemia with reduced osmotic fragility samples were tested for diagnosis of beta thalassaemia by determination of hemoglobin F% and hemoglobin A_2% with G7 high performance liquid chromatography (HPLC). Different types of haemoglobin were determined from whole blood samples by using isoelectric focusing gel electrophoresis (IEF). Silent beta thalassaemia carriers were identified according to mutation or deletion of beta globin gene in chromosome 11 from blood samples of adults who had hypochromic microcytic anemia with poikilocytosis, reduced MCV result, increased HbF% and HbA2% by using molecular method of single strand conformational polymorphism polymerase chain reaction (SSCP-PCR).

Molecular method

DNA was extracted from whole blood by using the DNA extraction kit containing lysing solution (131mM NH_4Cl and 0.9mM NH_4HCO_3), digestion solution (10mM Tris, 400mM NaCl, 2mM EDTA, 10% SDS and 3 µl of proteinase K). DNA was dissolved in 100 µl of TE buffer and stored in 4ºC refrigerator. DNA-extracted sample (15.5 µl) was mixed with 93 µl of EM solution (25mM $MgCl_2$, 10X buffer solution and distilled water) and divided into 15 PCR tubes containing 7 µl per each tube. This solution was mixed with 3 µl of 15 different primer solutions including TE (pH 8.0), dNTP and internal primers of human growth hormone gene: 429 bp (hGHF and hGHR).

DNA amplifications were carried out in the thermocycler under following conditions; Step 1 : 95°C for 10 minutes, Step 2 : 95°C for 1 minute, 63°C for 30 seconds, 72°C for 2 minutes (this Step 2 is repeated 10 times), Step 3: 95°C for 1 minute, 58°C for 30 seconds, 72°C for 2 minutes (this Step 3 is repeated 20 times), Step 4 : 72°C for 10 minutes.

Amplified product was carried out to 2% agarose gel electrophoresis at 150 volt for 30 minutes and stained with ethidium bromide to detect following 15 mutant genes according to 50 bp molecular base marker.

Mutant genes	Combination of primers	PCR size
-28CapA>G	BARMSFLC-28CapAGR	(128 bp)
CD 5-CT	CD5-CTF-B2xRII	(306 bp)
CD 8/9+G	BARMSF-nCD8/9+GR	(232 bp)
CD 15G>A	CD15GAF-B2xRII	(287 bp)
CD 17A>T	BARMSF-CD17ATR	(247 bp)
IVSI-1G>T	BARMSF-IVSI-1GTR	(293 bp)
CD 41/42-4del	B5UTL-41/42R	(315 bp)
CD 16-C	B5UTL-CD16-CR	(209 bp)
CD 26G>A(HbE)	B5UTL-NBEGAR	(139 bp)
IVSI-5G>C	BARMSF-IVSI-5GCR	(292 bp)
CD 35C>A	B5LUTL-CD35CAR	(308 bp)
CD 71/72+A	BInxF-n71/72+AR	(274 bp)
CD 6A>T(HbS)	BARMSF-CD6ATR	(225 bp)
-619del	619F-619R	(242 bp)
IVSII654C>T	nB2DF-n2M654R	(258 bp)

Data collection and statistical analysis

The results obtained throughout the study were recorded and all data were analyzed by using the computer based statistical package of statistical product and service solution (SSCP) version 11.5. The results were calculated by two samples t test with equal variances (Two sample Wilcoxon rank-sum - Mann-Whitney) test in this study.

RESULTS

Characteristics of subjects

A total of 516 healthy adults who are living in Yangon Division within the age of 16-45 years including 19 males and 497 females participated in this study. The mean age of the subject was 22.7±9.2 years in this study. The commonest age group was 15-20 years in both sexes (84% in males and 66% in females) and the least common age group was 41-45 years (5.3% in male and 1.8% in female) in this study (Table 1).

Table 1. Age and sex distribution of apparently healthy adults living in Yangon Region

Age range (years)	Male n(%)	Female n(%)	Total n(%)
15-20	16(84.2)	328(66)	344(66.7)
21-25	0	0	0
26-30	1(5.26)	91(18.3)	92(17.8)
31-35	1(5.26)	50(10)	51(9.8)
36-40	0	19(3.8)	19(3.8)
41-45	1(5.26)	9(1.8)	10(1.9)
Total	19(4)	497(96)	516

Table 2. Haematological parameters in iron deficiency anemia and beta thalassaemia minor (or) carriers

Haemato-logical tests	Normal value	Iron deficiency anemia	Beta thalassaemia minor
Hb (g/dl)	>12	11.07	11.1
MCV (μm^3)	>80	75	71.7
MCH (pg)	>26	23.9	22
OFT (%)	>90	79.2	57.3
HbA2 (%)	<3.5	2.8	48
HbF (%)	<1	1.1	2.5
Blood film examination	Normal blood film	Hypochromic microcytic with absence of target cells and aniso-poikilocytosis	Hypochromic microcytic anemia with presence of target cells and poikilocytosis, slight anisocytosis

Haematological parameters of subjects

Out of the 516 healthy adults, hypochromic microcytic anemia (mean Hb -11.1 g%) was detected in 103 subjects (20%) and 413 subjects (80%) had absence of anemia (mean Hb-13.2g%) in this study. Beta thalassaemia carriers were detected in 21 adults (4% in adult population) according to microscopic examination of blood film and reduced osmotic fragility test and increased HbA2% and HbF% by using high performance liquid chromatography (HPLC). Haematological parameters especially OFT, HbF% and HbA2% showed significant difference between iron deficiency anemia and beta thalassaemia carriers (p value <0.005) (Table 2).

Types of haemoglobin variants

Hemoglobin 'A' (normal hemoglobin) was detected in 416 adults (80.6%) of the total adult population comprising 13 male and 403 female subjects. HbEA was found in 88 out of 516 (17.1%) (6 males and 82 females) and HbEE was identified in 12 female subjects (2.3%) by using the iso-electric focusing gel electrophoresis test. Mean value of MCV, MCH and HbA2% showed significant difference among normal Hb (HbAA) and abnormal Hb types (HbEA, HbEE) ($p<0.000$). Other haemoglobin variants like HbS, HbC, HbH and HbBarts were not detected in adult population in this study.

Molecular diagnosis of beta globin gene mutation in silent beta thalassaemia carrier

Out of the 103 anemic adults, 63 subjects had low levels in MCV, MCH and OFT with more or less HbF% and HbA2%. Among them, haemoglobin subunit beta (HBB) gene mutation was detected in 52 adults (10% for adult population) by using polymerase chain reaction with single strand conformation polymorphism (PCR-SSCP) (Fig. 1).

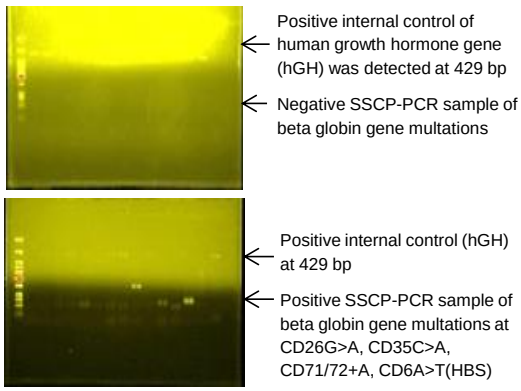

Fig. 1. Genetic mutations of beta globin gene (different genotypes of silent beta thalassaemia carrier) in 2% agarose gel electrophoresis according to the low molecular weight marker (50 bp)

Eleven subjects had absence of HBB gene mutation by PCR-SSCP method. The most common HBB gene mutation was CD26G>A (HbE). The other common five genetic mutations were CD35C>A, CD 6A>T(HbS), CD71/72+A, CD17A>T and IVSII 654C>T that were diagnosed as silent beta thalassaemia carriers (Table 3).

Table 3. Distribution of genetic mutation (genotypes) of silent beta thalassaemia carriers in adults living in Yangon Region

Genotype Code No	HBB gene mutation sites	Frequency	Positive %	Base pair (bp)
1	-28Cap A>G	1	1.6	128
2	CD 5-CT	1	1.6	306
3	CD8/9+G	0	0	232
4	CD15G>A	1	1.6	287
5	CD17 A>T	7	11.1	247
6	IVSI-1G>T	0	0	293
7	CD 41/42-4 del	5	7.9	315
8	CD16-C	5	7.9	209
9	CD26G>A (HbE)	42	66.7	139
10	IVSI-5G>C	0	0	292
11	CD 35C>A	33	52.4	308
12	CD 71/72+A	22	34.9	274
13	CD 6A>T (HbS)	33	52.4	225
14	-619 del	0	0	242
15	IVSII654C>T	6	9.52	258

Molecular screening for HBB mutation is a sensitive test and easy technique to detect the silent beta thalassaemia carriers among healthy adult population after testing of essential red blood cells parameters (Hb%, MCV, MCH, RDW) by automatic blood analyzer, OFT (osmotic fragility test) and morphology of red blood cells by microscopic blood film examination.

DISCUSSION

In this study, the commonest age group of both genders in apparently healthy adult population was 15-20 years (66.7%). The commonest HbAA type (normal Hb) was detected in 80.6% of adult population living in Yangon Region. HbEA was detected in 17.1% and HbEE was found in 2.3% of this population. HbE carrier prevalence rate was 19.09% (18.09% in HbEA and 1% in HbEE) in healthy school children (5-12 years) in Delta region of Myanmar.[9] In Myanmar, the prevalence of beta thalassaemia trait and HbE carrier was 1.18% and 19.9%, respectively, among couples and the risk for a severe thalassaemia in newborn ranges from 0.2-1.2 in the previous study.[10] If both parents have thalassaemia trait, their

children will have 1 in 4 chances to sickle cell anaemia developed to thalassaemia major.

Screening of thalassaemia trait in neonates and premarital adults are important for decreasing prevalence of thalassaemia major in our country. It may provide reduction in morbidity and mortality of mother and child, one of the main strategies of the National Health Plan in Myanmar. In this study, 21 healthy adults (4%) of beta thalassaemia carriers were detected by using haematological parameters and HPLC results. Silent beta thalassaemia carrier was identified in 52 adults (10%) in HBB gene mutation by PCR-SSCP. The most common HBB gene mutation was CD26G>A (HbE) (66.7%). The other common five genetic mutations were CD35C>A, CD6A>T (HbS), CD71/72 +A, CD17A>T and IVSII 654C>T.

Molecular screening for HBB mutation is a sensitive test and easy technique to detect the silent beta thalassaemia carriers among the healthy adult population after testing of essential red blood cells parameters (Hb%, MCV, MCH, RDW) by automatic blood analyzer and morphology of red blood cell by blood film examination. In Malay, three common β globin gene mutations (73.1%) are HbE (CD26G>A), IVS 1-5 (G>C), and IVSI-I (G>T). There are five common gene mutations in Chinese-Malaysians: CD41/42 (T-CTT), IVS2-654 (C>T), -28(A>G), CD17 (A>T) and CD71/72(+A).[11] In Chinese populations, the five most common genoypes in beta globin gene are codon CD41-42(-TCTT), IVS-2-654(C>T), 28(A>G), CD17 and CD71-72 (+A) by multiplex PCR.[12] In Myanmar, six point mutations were identified in the G-T at IVS-1 position, the G-C at IVS-1 position and deletions of TCTT codon for 85% of alleles in beta thalassaemia major patients.[13]

Twelve new genetic mutations were detected in thalassaemia major by using ARMS-PCR (Amplification Refractory Mutation System). Among 18 different mutations which were identified, 14 samples were β thalassaemia homozygous (β0) and 4 samples, HbE β-tha Heterozygous (β+).[14] Genotyping of Myanmar thalassaemic children attending Yangon Children Hospital detected five common beta thalassaemia mutants (CD17A-T, IVS1-1GT, IVS1-5G-C, CD41/42-TCTT 4 bp deletion and hemoglobin E.[15] In this study, the six common genotypes of silent beta thalassaemia in adult population are nearly the same as common genotypes of Chinese-Malaysian populations in Malaysia and Chinese populations in China.

In this study, the six common genotypes of beta thalassaemia carriers are significantly different in common genotypes of beta thalassaemia patients in Myanmar. The classical phenotype of heterozygous beta thalassaemia could be modified by a number of environmental and genetic interacting factors such as coinheritance of alpha thalassaemia, mild beta thalassaemia mutation, co-transmission of delta thalassaemia and presence of a silent mutation of beta globin gene.[16]

Different thalassaemia genotypes have great variability of clinical severity and patients with identical genotype can have different levels of severity. Beta thalassaemia/HbE genes leads to homozygous beta thalassaemia that is a major thalassaemic syndrome in Asia Region. Therefore, premarital screening for thalassaemia is standard practice and National data are available for an effective control program in Asian countries.[17] A screening program is needed in adult populations to be supported by public health education and advanced rapid and accurate laboratory diagnosis. Screening of silent thalassaemia carriers and providing the proper health education in adult populations are part of the main strategies for prevention and control of new cases of thalassaemia major in Myanmar.

Competing interests

The authors declare that they have no competing interests.

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