Quantification of hemoglobin peptides in Beta-thalassemia patients using tandem mass spectrometry for future national screening program

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Background
β-thalassemia is a hereditary blood disorder characterized by reduced or absent synthesis of the β chains of hemoglobin resulting in variable disease severity. The high carrier rate of thalassemia in Egypt makes it a priority genetic disease for prevention programs through detection of new cases and screening for carriers.

Patients and methods
In this study, for the first time in Egypt, tandem mass spectrometry (MS/MS) is used to distinguish patients with β-thalassemia from carriers and controls by calculation of α/β globin peptides ratio, as a contributory step in the management of this disease. The study included 40 patients with β-thalassemia referred from the Hereditary Blood Disorders Clinic, National Research Centre, 32 β-thalassemia carriers (parents of cases), and 34 healthy normal participants of matching age and sex. Dried blood spots from all participants were analyzed using MS/MS, followed by confirmatory molecular analysis.

Results
The results of MS revealed that α T1/β T1 globin peptides ratio is the most informative ratio that could be used to differentiate between β-thalassemia cases, carriers, and normal participants. The mean value of α T1/β T1 ratio for the studied cases was 4.24±0.97 (4.41±1.092 for thalassemia major and 3.83±0.88 for thalassemia intermedia), 1.93±0.30 for carriers, and 1.20±0.06 for controls, with statistical significant difference (P<0.001).

Conclusion
In conclusion, distinguishing patients from carriers and controls through tandem mass technology can serve as a cost-effective tool for national screening program for thalassemia in Egypt.

Keywords:
β-thalassemia, dried blood spot, Egypt, tandem mass spectrometry, globin ratio

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Introduction

Thalassemias are the most common congenital hematological disorders worldwide. They result from inherited defect in hemoglobin synthesis and occur more frequently in the Mediterranean region, Southeast Asia, and West Africa (Weatherall, 2011). It is estimated that ~7% of the world population carries a globin-gene mutation that is mostly inherited as an autosomal recessive trait (Weatherall and Clegg, 2001).

β-thalassemia is a group of inherited blood disorders, resulting from reduced or absent synthesis of the β chain of hemoglobin. Global annual incidence is estimated at one in 100 000 (Galanello and Origa, 2010). This condition is commonly found in the so-called 'thalassemia belts,' which stretch from the Eastern Mediterranean through the Middle East (Putri *et al.*, 2018). In β-thalassemia, the imbalance in α/β globin peptide ratio with excessive α-globin peptides causes oxidative damage to membrane lipids and proteins of the red cell in the form of irreversible hemi chromes as well as increases intracellular calcium ultimately resulting in significant increase in destruction of the red blood cells (RBCs) and anemia (Rund and Rachmilewitz, 2005). The clinical severity of the disease correlates with the degree of this imbalance and the size of free α-chain pool (El Kamah and Amr, 2015).

Patients are managed through supportive lifelong blood transfusion, risking the hazards of iron overload, and splenectomies are also performed in case of hypersplenism (El-Beshlawy and Youssry, 2009). In the absence of curative therapies so far, apart from bone marrow transplantation, which is limited by

the availability of specialized centers and compatible donors, disease prevention becomes very important.

β-thalassemia is characterized by a high mutation rate and the presence of genetic modifiers. More than 200 β globin mutations are reported; they differ significantly among different geographical regions and ethnic groups. The vast majority are point mutations within the gene or its immediate flanking sequences (Hussein *et al.*, 1993; Omar *et al.*, 2005; El Kamah and Amr, 2015).

Different studies have exploited on the convenience of tandem mass spectrometry (MS/MS) for hemoglobinopathy screening, including HbE, HbQ, HbS, HbC, and β-thalassemia by using dried blood spot (DBS), and MS/MS was proved to be sensitive, quantitative, accurate, and suited to high-volume testing (Daniel *et al.*, 2005; Boemer *et al.*, 2011; Yu *et al.*, 2015).

In Egypt, β-thalassemia is the most common chronic hemolytic anemia (85.1%). The carrier rate varies from 5.3 to more than or equal to 9%, with an estimate of 1000/1500 million live births per year to have thalassemia, creating a social and financial burden for the patients' families and the Egyptian government (El-Beshlawy *et al.*, 1999; Clarke and Higgins, 2000). Therefore, a prevention program is highly demanded (El-Hashemite *et al.*, 1997).

Many previous studies have been concerned about the existence of a prevention program (El-Beshlawy *et al.*, 1999; Omar *et al.*, 2005; Hussein *et al.*, 2007; El-Shanshory and Hagag, 2014). The current study aims at providing a cost-effective reliable screening program through successfully differentiating patients with β-thalassemia from carriers and controls using MS/MS for the first time in Egypt.

Patients and methods

Patients

A total of 106 samples were studied over a period of 1 year; including 40 patients with β-thalassemia (16 cases with β-thalassemia intermedia and 24 with β-thalassemia major), whose ages ranged from 3 months to 18 years; 32 β-thalassemia carriers (parents of some of the studied cases), whose ages were between 21 to 51 years; and 34 healthy normal participants of matching age and sex to the studied patients.

Clinically suspected β-thalassemia cases were recruited from the Hereditary Blood Disorders Clinic, at the Medical Center of Excellence, National Research Centre. They were confirmed using capillary electrophoretic (CE) separation. Ethical approval following the regulations of Helsinki-ethical principles for medical research involving human participants through the institutional review board at the National Research Centre was obtained, and patients or their guardians before inclusion in the study signed an informed consent.

DBSs were collected on filter paper (Whatman, 903) from all cases, carriers, and controls to be stored at 4°C for mass analysis. Genotyping analysis was performed for cases as a confirmatory test.

Methods

Capillary electrophoresis

It was done by using Minicap Hemoglobine (PN 2207) kit. The separation is accomplished in a buffer-filled fused silica capillary tube 10–100 cm in length. The ends of the capillary are placed in two buffer containers in contact with the anode and the cathode of the power supply at a potential of 1–30 KV. The retention time of a molecule is the result of its own electrophoretic mobility plus the buffer's electroosmotic flow. This flow plays an important role in CE. It results from the negative charge on the silica surface of the capillary and acts like a pump delivering the molecules toward the cathode through an on-line detector, which is mostly an ultraviolet spectrophotometer (Ong *et al.*, 1992).

Tandem mass spectrometry

In this technique, there was a selection of specific peptide ions (precursor ions) with prespecified (mass/charge) m/z values in the first quadrupole (Q1), followed by a fragmentation process in a collision cell (Q2) and targets the specific selected ion (daughter or product ions). Finally, the produced fragment ions were monitored on the third quadrupole (Q3) for construction of the multiple reaction monitoring (MRM) scan mode, which is a very sensitive, specific detection, and/or quantification for targeted analytes (Aebersold and Mann, 2003; Domon and Aebersold, 2006).

Sample preparation and digestion procedure

With the usage of trypsin, the α-globin chain was cleaved into fourteen proteospecific peptides (T1–T14) and the β-globin chain was cleaved into fifteen proteospecific peptides (T1–T15) (Daniel *et al.*, 2005).

T1 (VLSPADK), T3 (AAWGK) and T1 (VHLTPEEK) and T2 (SAVTALWGK) were used

as the most informative proteospecific peptides and as precursor ions for α- and β-globin chain, respectively, according to Yu *et al.* (2015, 2017). These precursor ions were subjected to collision-induced energy to produce product ions for MRM construction (Table 1).

For the preparation and digestion of the samples, we followed the method of Yu *et al.* (2017). DBS card was punched into 96-well microplates, and 200 μl of deionized water was added to each well. After gently rotating for 60 min, 100 μl of aliquots was transferred to a fresh 96-well microplate. Then, 30 μl of acetonitrile and 10 μl of formic acid (12 g/l) were added to each well before vigorous mixing to denature the proteins. After incubation at room temperature for 5 min, 10 μl of a (N-tosyl-L-phenylalanine chloromethyl ketone) TPCK-treated trypsin solution (5 mg/ml with 1 mol/l ammonium bicarbonate) was added for digestion. All samples were incubated at 37°C for 2 h, and 12 μl of the digested solution was diluted with 108 μl of acetonitrile/deionized water (1:1) containing 1.2 g/l formic acid.

Tandem mass spectrometry

MS/MS analysis was performed using waters Xevo triple quadruple MS (Milford, Connecticut, USA) coupled to waters Acquit UPLC system. A scheduled MRM acquisition method was constructed using manually optimized of cone voltage and collision energy using a sample of patients with β-thalassemia. The cone voltages of αT1 and αT3 were 32 and 30 V, whereas in case of βT1 and βT2 were 19 and 32 V, respectively. The collision energies were 40 eV for both αT1 and αT3; however, for βT1 and βT2, they were 30 and 25 eV, respectively. All chromatograms were analyzed with MassLynx V4.1 software.

Calculations of α/β globin peptides ratio

To distinguish between cases, carriers, and controls, the α/β globin peptides ratios were calculated based on the integrated peak area of the selected MRM transitions.

Peptide peak areas were calculated by integrating the smoothed and combined chromatogram from all corresponding two MRM channels, using The MassLynx V4.1 software. Then the results have been exported to Excel, and area ratio (α/β) calculations for two MRM channels have been performed.

Molecular analysis

Genomic DNA was extracted from peripheral blood samples from patients using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Using reverse hybridization strip assay (Viennalab), β-thalassemia mutations were identified by PCR amplification using biotinylated primers.

Statistical analysis

Data were statistically described in terms of mean±SD, median and range, or frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Mann–Whitney *U* test. Accuracy was represented using the terms sensitivity and specificity. Receiver operator characteristic (ROC) analysis was used to determine the optimum cutoff value for mass value to discriminate β zero from β+. Two-sided *P* values less than 0.05 were considered statistically significant. All statistical calculations were done using computer program IBM SPSS (Statistical Package for the Social Science; IBM Corp., Armonk, New York, USA), release 22 for Microsoft Windows.

Results

The study included 40 patients with β-thalassemia (16 males and 24 females) with age range from 3 months to 18 years old. Sixteen patients had β-thalassemia intermedia (TI) and 24 were diagnosed as having β-thalassemia major (TM). Positive parental consanguinity was present in 8/16 (50%) of the patients with β-thalassemia intermedia and in 14/24 (58%) of the β-thalassemia major (Table 2).

The hematological data of the patients with thalassemia major were as follows: the mean RBCs count was 3.70±0.162, white blood cell count was 7.42±2.5 and hemoglobin level was 5.8±0.8 g/dl. However, in case

Table 1 The precursor and product ions of the most informative peptides of α-globin chain (αT1-αT3) and β-globin chain (βT1-βT2)

Proteospecific peptides	Precursor *m/z*	Product *m/z*	Amino acid sequence
αT1	729.3	430.2	VLSPADK
αT3	532.3	204.1	AAWGK
βT1	952.3	502.3	VHLTPEEK
βT2	932.3	390.2	SAVTALWGK

Table 2 Demographic data among 40 patients with β-thalassemia

	Demographic data number of cases (%)	
Sex		
Male	16/40 (40)	
Female	24/40 (60)	
Age	3 months-18 years	
Types of β-Thalassemia	Thalassemia intermedia	Thalassemia major
Number of cases	16/40 (40)	24/40 (60)
Consanguinity	8 +ve (50) 8 -ve (50)	14 +ve (58.3) 10 -ve (41.7)

of thalassemia intermediate, mean RBC count was 4.54±0.260, white blood cell count was 8±3.3, and hemoglobin level was 8.6±1.2 g/dl. Hepatosplenomegaly was detected in 18/24 (75%) patients with TM, whereas only splenomegaly was detected in 6/24 (25%). Of 16 patients with TI, 11 (68.7%) had hepatosplenomegaly, whereas three (18.7%) cases had splenomegaly (Table 3).

The results of statistical analysis of electrophoretic separation showed a significant *P* value less than 0.001 between TI and TM patients for HbA, HbA2, and HbF levels. In the same line, *P* value for HbA, HbA2, and HbF levels between cases, carriers, and controls showed also significant value (<0.0001) (Table 4).

The patient sample was used to optimize the cone voltage of each proteospecific peptides to get the highest peak with high sensitivity and specificity for precursor ions, and then the same sample was used to optimize the collision energy to get the daughter ions for each precursor for MRM mode (Table 5).

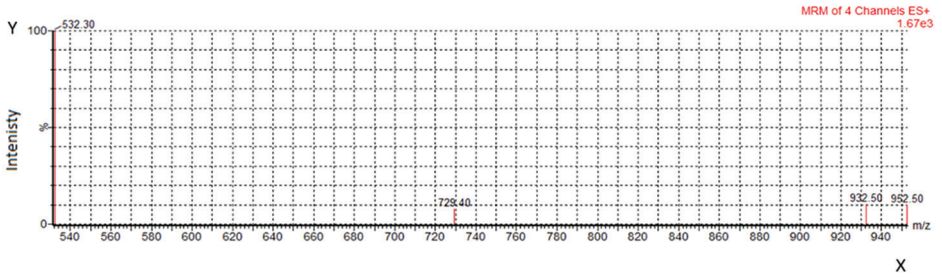
Fig. 1 shows the chromatography of MRM scan mode for four channels of αT1, αT3, βT1, and βT2 globin peptides. The values on the chromatographic picture represent the parent ions for each globin peptide.

The mean values of αT3/βT2 and αT1/βT1 globin peptides ratio in thalassemia major equal 43.53±29.4 and 4.41±1, respectively. In case of thalassemia intermediate, the mean values of the same globin peptides were 40.52±17.77 and 3.83±0.88, respectively, with no significant difference between two groups of thalassemia. The mean values of that globin peptides ratio in cases were higher than carriers and controls, and in the same direction, the mean values of carriers were higher than controls, with *P* value less than 0.001 (Table 6).

Table 7 shows pairwise comparison between the *P* values of MS/MS results of case–control, case–carrier, and control–carrier of the studied groups, which showed significant difference with αT1/βT1 peptides ratio in all groups. On the contrary, αT3/βT2 shows significance only for case–control (*P*<0.001), but no significance for case–carrier (*P*<0.21) and for control–carrier (*P*<0.71) groups.

According to Table 8, there was no significant difference in mass results (*P*<0.328) between positive and negative consanguinity.

Figure 1



MRM scan mode of four channels for αT1 (729.3–430.2), αT3 (532.3–204.1), βT1 (952.3–502.3), and βT2 (932.3–390.2). Y-axis, intensity; X-axis, mass/charge (*m*/*z*). MRM, multiple reaction monitoring.

Table 3 Clinical and hematological data of studied patients

	Thalassemia major (*n*=24)	Thalassemia intermediate (*n*=16)
Age (year) (mean±SD)	0.3-2.3 (1±0.6)	3-18 (6.3±4.7)
Red blood cells (million cell/μl) [range (mean±SD)]	3.5-4 (3.70±0.162)	4.4-5 (4.54±0.260)
Hemoglobin (g/dl) [range (mean±SD)]	4.6-7 (5.8±0.83)	7.6-12 (8.6±1.23)
White blood cells (cells/μl) [range (mean±SD)]	2.4-11.4 (7.42±2.5)	2.8-12.6 (8±3.3)
Regular blood transfusion [*n* (%)]	24 (100)	0
Did not receive blood transfusion [*n* (%)]	0	16 (100)
Hepatosplenomegaly [*n* (%)]	18 (75)	11 (68.7)
Splenomegaly [*n* (%)]	6 (25)	3 (18.7)
With no hepatosplenomegaly [*n* (%)]	0	2 (12.5)

Table 4 Mean values of hemoglobin A, A2, and F using capillary electrophoresis

	Cases (mean±SD)			Carriers (mean±SD)	Controls (mean±SD)	*P*
	Thalassemia major	Thalassemia intermediate	*P*			
HbA (%)	30±23	60±31	<0.001	94.33±1.43	97.55±0.5	<0.0001
HbA2 (%)	2±0.62	8±2	<0.001	3.71±0.4	1.76±0.56	<0.0001
HbF (%)	68±23.5	34±32.8	<0.001	1.6±0.28	0.58±0.18	<0.0001

Mean ratio of αT1/βT1 for β° equals 5.033±0.6, whereas in case of different genotypes for β⁺, the means ranged from 3.1±0.58 to 3.8±0.41. The result showed that β° genotype had a higher value than other mutations (Table 9).

We performed ROC analysis to identify the cutoff level of the marker αT1/βT1 peptide ratio to differentiate between β° and β⁺ thalassemia. The best cutoff level of the marker was 4.215, which achieved specificity of 72 and sensitivity of 78 (Fig. 2).

Discussion

In a clinical laboratory setting, routine analysis of hemoglobinopathies is carried out by using electrophoretic methods or ion exchange HPLC procedures. MS has been used for analysis of Hb variants for the past three decades (Wada *et al.*, 1981). Currently, 1500 abnormal Hb variants have been identified, and this complexity might lead to ambiguous HPLC results because of limited resolving power, which in addition to the possibility of uninterpretable or false-positive results, even for common Hb variants such as HbS, HbD, and HbG (Greene *et al.*, 2015).

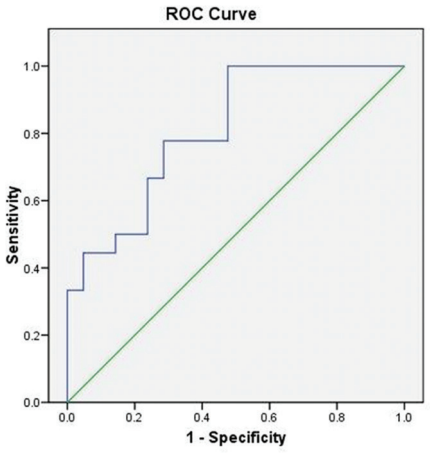
On the contrary, MS/MS is a technique to identify molecules based on their mass (molecular weight) to charge ratio. The strong advantage of the technique is that it uses minimal specific binding reagent for the molecules of interest. The simple analytical principle enables less interference and more accurate identification. The procedure is automated, highly sensitive, quantitative, accurate, and suited to high-volume testing. In addition, MS/MS is a reference technique in all newborn screening laboratories (Domon and Aebersold, 2006; Cleona *et al.*, 2009).

In the current study, the α/β globin peptides ratios of 106 DBSs were calculated by using MS/MS technique to differentiate patients with β-thalassemia from carriers and controls for future national screening program for thalassemia in Egypt.

β-thalassemia phenotypes are variable, ranging from the severe transfusion-dependent thalassemia major to the mild form of thalassemia intermedia (Galanello and Origa, 2010). Patients with the major form of the disease have severe anemia, microcytic and hypochromic anemia, hepatosplenomegaly, and usually come to medical attention within the first 2 years of life (Borgna-Pignatti *et al.*, 2004). In the present study, 24 (60%) patients with age range 0.3–2.3 years had severe anemia with low Hb level less than 7 g/dl and low RBCs count. Eighteen of them had hepatosplenomegaly, and the rest had splenomegaly. These patients were receiving regular blood transfusion at a rate of 1–2 transfusions/month and diagnosed as thalassemia major.

The clinical phenotypes of thalassemia intermedia are between those of thalassemia minor and major, where some patients are asymptomatic until adult life, whereas others are symptomatic from as young as 2 years of age. They show mild to moderate anemia and a hemoglobin level ranging between 7 and 10 g/dl, which is sustainable without the need for regular transfusion

Figure 2



The ROC test. ROC, receiver operator characteristic.

Table 5 Mass optimization conditions for multiple reaction monitoring of the most informative peptides of α and β-globin chains

Tryptic peptides	MRM transition (*m/z*)	Cone voltage	Collision energy
α			
αT1	729.3/430.2	32	40
αT3	532/204.1	30	40
β			
β T1	952.3/502.3	19	30
β T2	932.3/390.2	32	25

MRM, multiple reaction monitoring.

Table 6 The mean values of mass results of ratio globin peptide chains for αT3/βT2 and αT1/βT1 for cases, carriers, and controls

	β-thalassemia cases			Carriers (mean±SD	Controls (mean±SD)	*P*
	TM	TI	All cases (mean±SD)			
αT3/βT2	43.53±29.4[a]	40.52±17.77[a]	40.59±23.23[a]	30.46±20.65	24.86±18.03	<0.001
βT1/βT1	4.41±1.092[a,b]	3.83±0.88[a,b]	4.24±0.970[a,b]	1.93±0.30	1.20±0.06	<0.001

TI, thalassemia intermedia; TM, thalassemia major. [a]Statistically significant difference in comparison to control group. [b]Statistically significant difference in comparison to carrier group.

therapy (Galanello and Cao, 1998; Taher *et al.*, 2006)). Sixteen (40%) of our patients were clinically classified as thalassemia intermediate, with age range from 3 to 18 years; hemoglobin range was 7.6–12 g/dl, and they did not receive any blood transfusion (Table 3).

Traditionally, electrophoresis has been the method of choice for identification and quantification of variant Hbs. The distinguishing finding in β -thalassemia is a hemoglobin electrophoresis with the finding of elevated HbA2 and HbF.

The Hb pattern in β-thalassemia varies according to β-thalassemia type. In β-thalassemia, it is characterized by the lack of β globin chain synthesis, and HbA is almost absent with predominance of HbF and HbA2. In β^+-thalassemia homozygotes with a residual variable β globin synthesis or β°/β^+ compound heterozygotes, the Hb pattern shows HbA between 10 and 30%, HbF in the order of 70–90%, and HbA2 of 2–5% (Cao and Galanello, 2010). Among our cohort of patients with BTM, HbA ranged from 0 to 60%, HbA2 1.5–3%, and HbF 39–98%.

The hematological phenotype of thalassemia intermedia is very wide, ranging in severity from that of the β-thalassemia carrier state to that of thalassemia major (El Kamah *et al.*, 2003; Galanello and Origa, 2010). Sixteen patients with thalassemia intermediate had HbA level between 14 and 90%, HbA2 level from 4 to 9%), and HbF from 1.5 to 80% (Table 4).

MS/MS is already effectively used for screening of inherited metabolic disorders (Schulze *et al.*, 2003; Scaturro *et al.*, 2013); hence, different strategies have been assessed to identify variants using either whole-protein scan to measure the masses of intact globin chains (Domon and Aebersold, 2006) or analysis of tryptic peptide fragments (Daniel *et al.*, 2005).

According to Yu *et al.* (2015, 2017), T1 (VLSPADK), T3 (AAWGK) and T1 (VHLTPEEK) and T2 (SAVTALWGK) were used as the most informative proteospecific peptides (precursor ions) for α- and β-globin chain respectively. Therefore, cone voltages for these peptides were optimized using patient blood samples.

By using the same blood sample, collision energies for product ions were optimized to obtain the highest intensity peaks to construct MRM scan, for quantification, and the net results are shown in Table 5 and Fig. 1.

The mean level of αT3/βT2 ratios of the patients with β-thalassemia and carriers showed overlapping of both levels due to the wide range of SD. However, the mean level of αT1/βT1 globin peptides ratio in patients with β-thalassemia can be differentiated from that of the β-thalassemia carriers (Table 6). Moreover, the pairwise comparison between the *P* values of mass results of case–control, case–carrier, and control–carrier gave a significant difference among the three groups ($P<0.001$) with αT1/βT1 ratio (Table 7), and accordingly, αT1/βT1 ratio is the most informative one that could be used to differentiate among β-thalassemia cases, carriers, and normal participants. Our results differ from Yu *et al.* (2017), who used the ratio αT3/βT1 in their calculations and stated that the α/β ratio calculated from αT3 and βT1 showed higher precision; therefore, the peptides (αT3 and βT1) were used for calculation.

In patients with β-thalassemia, mutations in the β-globin gene decreased the expression of β-globin subunits. Subsequently, this reduction resulted in the accumulation of excess α-globin chains. Therefore, the α-globin versus β-globin ratio increases compared with normal individuals (Daniel *et al.*, 2005; Boemer *et al.*,

Table 7 Pairwise comparison between *P* value of mass results of cases-controls, cases-carriers, and controls-carriers of the studied groups

	Case vs. control	Case vs. carrier	Control vs. carrier
αT3/βT2	<0.001	<0.21	<0.71
αT1/βT1	<0.001	<0.001	<0.001

Table 8 Relation between mass results and consanguinity

Consanguinity	Number of patients	Mass results of αT1/βT1 peptides ratio (mean±SD)	*P*
Negative	18	4.371±0.8712	0.328
Positive	22	4.01±1.1082	

Table 9 Relation between mass results and gene severity

Gene of mutations	Severity	Number of patients	MS/MS results of αT1/βT1 peptides ratio (mean±SD)
Homozygous IVS I.1	β°	19	5.033±0.6
Heterozygous IVS I.1/IVS II.1	β°	2	4±0.54
Heterozygous IVS I.110/cod 6	$B^\circ\ \beta^+$	1	3.8
Homozygous Cod 27G >T	β^+	2	3.5±0.3
Homozygous IVS I.110	β^+	4	3.8±0.41
Homozygous IVS I.6	β^+	12	3.1±0.58

MS/MS, tandem mass spectrometry.

2011). Such imbalance was clearly observed in mass results among cases (4.24±0.97), carriers (1.93±0.3), and controls (1.20±0.06) with a significant difference (P<0.001). Our results correlate well with the study of Yu *et al.* (2015, 2017), which concluded that increased α/β globin ratios were observed in almost each type of β-thalassemia, and the degree of disease severity correlates well with the degree of this imbalance and the size of free α-chain pool (Table 6).

We did not find a significant difference in peptide globin ratios of mass results between thalassemia intermediate (3.83±0.88) and thalassemia major (4.41±1), because the ratio depended on the genotype picture of mutations, which included homozygous and heterozygous of β^0 and β^+, and the two thalassemia types are a mixture of them (Table 6).

Regarding the effect of consanguinity on mass results, the P value was less than 0.328, with no significant difference in mass results (Table 8). The mean of αT1/βT1 globin ratio in positive consanguinity was 4.01±1.1082 and in the negative was 4.371±0.8712. The small numbers of cases inside each group did not give a clear answer about the relation between mass results and consanguinity.

The molecular results confirmed our aim for using MS/MS as a cost-effective technique. We found that the mass results were matched with the severity of types of β-thalassemia (Table 9), where the range of α/β globin ratio in 21 patients with β^0-thalassemia was 4±0.54–5.033±0.6, which is higher than that of the 18 patients with β^+-thalassemia (3.1±0.58–3.8±0.41). This is in line with the previous studies and with the meaning of β-thalassemia mutation types because α/β globin ratio is reverse proportional with the expression of β-globin chain.

The cutoff level for the α/β ratios was determined by ROC curve to differentiate β^0 (3.6–6) from β^+ (2.4–4), and it was 4.21, with specificity of 72 and sensitivity of 78 (Fig. 2).

β-thalassemia is considered one of the serious health problems and the commonest hemoglobinopathy in Egypt, which creates a burden not only on health system but also on the affected families and children who become vulnerable to emotional, social, psychological, and behavioral problems (Adam *et al.*, 2017).

Conclusion

In conclusion, MS technique has proved its efficiency in the field of Hb analysis since 1981 (Wada *et al.*, 1981). The current study confirmed the ability to distinguish patients with β-thalassemia from carriers and controls using MS/MS providing a cost-effective means for a preventive program for the exhausting problem of β-thalassemia in Egypt.

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Conflicts of interest

There are no conflicts of interest.

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