δβ-Thalassemia Trait

How Can We Discriminate It From β-Thalassemia Trait and Iron Deficiency Anemia?

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Key Words: δ; β; Thalassemia; Microcytic anemia; Differential diagnosis

ABSTRACT

Objectives: To analyze the differences not only in classic hematologic parameters but also in RBC subpopulations among δβ-thalassemia trait (δβ-TT), β-thalassemia trait (β-TT), and iron deficiency anemia (IDA) and to evaluate the role of fetal hemoglobin (HbF) in elevated RBC distribution width (RDW).

Methods: Samples from 553 patients with microcytosis (74 δβ-TT, 272 β-TT, and 207 IDA) were run on an Advia 2120i analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY). Classic hematologic parameters and RBC subpopulations were assessed. The correlation between HbF and RDW in patients with thalassemia (both β and δβ) was evaluated. An independent sample t test was used to compare classic hematologic parameters and RBC subpopulations among β-TT, IDA, and δβ-TT and receiver operating characteristic curves performed in the significant comparisons.

Results: RDW was significantly higher in δβ-TT compared with β-TT (18.79% vs 16.04%, P < .001), as was mean corpuscular volume (66.39 vs 64.82 fL, P < .001), mean corpuscular hemoglobin (20.73 vs 20.04 pg, P < .001), and mean corpuscular hemoglobin concentration (31.16 vs 30.66 g/dL, P = .03). Pearson coefficient showed a good correlation between HbF and RDW. The values obtained for all the parameters were significantly different (P < .001) between patients with thalassemia (β and δβ) and IDA.

Conclusions: RDW is the best parameter to discriminate δβ-TT from β-TT. The degree of anisocytosis in patients with β-TT and δβ-TT is strongly correlated with HbF.

Since iron deficiency and thalassemia are the most common causes of microcytic anemia and the clinical management of both pathologies is quite different, the discrimination between thalassemic and nonthalassemic microcytosis has important implications.1 Iron deficiency anemia (IDA) may result from menstrual loss in childbearing-age women, inadequate iron intake, or chronic blood loss in the gastrointestinal tract in elderly patients.2 Impaired globin chain synthesis and decreased hemoglobinization can lead to microcytic anemia in patients with thalassemia.3 Most molecular mechanisms of β-thalassemia are point mutations (single-base substitution) or insertions or deletions involving several nucleotides, while gene deletion is less common. Deletions affecting the β-globin gene cluster result in the syndromes of β-thalassemia, hereditary persistence of fetal hemoglobin (HPFH), δ-thalassemia, and γδβ-thalassemia. The δβ-thalassemia trait (δβ-TT) results from the deletion of...
β and δ genes and is characterized by an elevation of fetal hemoglobin (HbF) with normal values of hemoglobin (Hb) A₂. Patients with a heterozygous condition are asymptomatic or develop mild anemia, whereas homozygotes usually have thalassemia intermedia.

Despite being less frequent than the β-thalassemia trait (β-TT), δβ-TT is not a rare condition in some geographical areas and sometimes may be misdiagnosed, so it is crucial to have efficient tools to distinguish both pathologies to give the patients proper genetic counseling. Classic RBC parameters are similar in both entities, with the exception of RBC distribution width (RDW). HbF is restricted to a few erythrocytes (F cells) in normal human adults, but the reasons for this fact remain unknown. Heterogeneous distribution of HbF among RBCs may lead to two different RBC populations and, consequently, to an elevated RDW in patients with δβ-TT. According to RDW, patients with δβ-TT can be discriminated from those with β-TT. However, no statistical correlation between HbF and RDW has been reported to date. Moreover, since patients with IDA also have elevated RDW, different tools for a presumptive diagnosis can be very helpful if the iron study results are not ready or inconclusive.

It is sometimes inappropriate to use classic hematologic parameters to discriminate patients with IDA from those with thalassemia or anemia of chronic disease. Because of that, RBC subpopulations in β-TT and IDA have been evaluated in several studies. Both pathologies have typical patterns in the histograms provided by hematologic analyzers of different manufacturers. In addition, linear discriminant functions based on hemogram data have been proposed to differentiate between IDA and thalassemia, so the samples can be selected for further genetic analysis. However, none of the mentioned studies included patients with δβ-TT, so the question of whether RBC subpopulations are able to discriminate patients with δβ-TT still remains unclear. The aims of our study were (1) to analyze the differences not only in classic hematologic parameters but also in RBC subpopulations among δβ-TT, β-TT, and IDA; (2) to clarify the role of HbF in elevated RDW; and (3) to evaluate the diagnostic performance of the parameters in the three types of microcytosis.

Materials and Methods

Study Samples

Over a 13-month period (March 2012–April 2013), 553 individual unique cases of microcytosis (mean corpuscular volume [MCV] <80 fL) recruited in the Central Laboratory of Madrid, Spain, were initially included in the study: 74 with δβ-TT, 272 with β-TT, and 207 with IDA. All samples were collected in K3-EDTA anticoagulant (Vacuette; Greiner Bio-One, Alphen aan de Rijn, the Netherlands), and a CBC and an iron panel (serum iron, ferritin, transferrin, and transferrin saturation index [TSI]) were performed in all samples. Patients with IDA with Hb levels less than 90 g/L were not included because in daily practice, they are not considered to have the thalassemia trait. To increase the clinical applicability of the study, patients with thalassemia and concomitant iron deficiency (ferritin <20 ng/mL and/or TSI <20%) were not excluded from the analysis.

Analytical Methods

The CBC was performed with the Advia 2120i analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY) within 6 hours of collection. Ferritin, transferrin, and TSI were measured by chemiluminescence immunoassay in the Advia Centaur (Siemens Medical Solutions Diagnostics).

HbA₂ and HbF levels were determined by high-performance liquid chromatography on the HA-8160 analyzer (Menarini Diagnostics, Florence, Italy). Patients with increased HbA₂ levels (>3.4%) were considered to have β-TT, whereas diagnosis of δβ-TT was made if HbF was more than 3% and HbA₂ was 3.4% or less. In doubtful cases, a multiplex ligation-dependent probe amplification (MLPA) study was carried out to screen for deletions in the human β-globin gene cluster. The MLPA is a comparative method based on the quantitative amplification and a subsequent fragment analysis of multiple probes hybridized across a region of interest. Since probe amplification can be achieved only when target DNA is in the sample, this method allows for a genetic profile showing the copy number variation of those targets in a patient’s genome. Here we used a commercial kit (MLPA kit P102-B2 HBB; MRC-Holland, Amsterdam, the Netherlands) that contains 28 probes designed to detect copy number changes in the hemoglobin β locus, from 1 Mb upstream the locus control region to 10 Kb downstream of β-globin gene. MLPA reactions were performed according to the manufacturer’s instructions and as previously described. Amplification products were separated by capillary electrophoresis on an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA). GeneMapper version 3.7 (Applied Biosystems) was used for size calling, and the data obtained were analyzed with Coffalyser software (MRC-Holland). Five normal DNA samples, with normal RBC indices, were used as healthy controls for MLPA reactions. Absolute value of HbF was obtained by multiplying total Hb levels by the percentage of HbF.

The following variables obtained from the Advia 2120i analyzer were assessed in the three groups of patients: RBC count, Hb levels, MCV, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), RDW, percentage of macrocytic RBC (%MACRO), percentage of microcytic RBC (%MICRO), percentage of hyperchromic RBC (%HYPER), and percentage of hypochromic RBC (%HYPO).
To analyze the impact of HbF in the RDW, three subsets of patients with thalassemia were considered: β-TT with HbF less than 2%, β-TT with HbF more than 2%, and δβ-TT.

A second step analysis was made to validate the parameters with higher discriminant efficiency (area under the curve [AUC] > 0.8) between δβ-TT and β-TT. Therefore, from June 2013 through March 2014, we prospectively collected all δβ-TT and β-TT cases diagnosed in our laboratory and evaluated whether the best cutoff for those parameters identified the type of thalassemia. For this second step, 218 β-TT and 41 δβ-TT new individual cases were evaluated.

**Statistical Analysis**

An independent sample *t* test was used to compare classical hematologic parameters and RBC subpopulations among β-TT, IDA, and δβ-TT. Bonferroni correction was used to counteract the problem of multiple comparisons, and *P* values less than .05 were considered statistically significant. Receiver operating characteristic (ROC) curves were plotted for those parameters that showed significant differences. The AUC of the parameters was used to evaluate their diagnostic performance. A cutoff was selected for RDW to discriminate β-TT from δβ-TT. The Pearson coefficient was estimated to assess the correlation between HbF and RDW and a one-way analysis of variance test was performed to compare the values of RDW in β-TT with HbF less than 2%, β-TT with HbF more than 2%, and δβ-TT. SPSS version 19.0 for Windows (SPSS, Chicago, IL) was used for statistical analysis of the results.

**Results**

The reliability of the results is guaranteed with daily internal quality control (provided by the manufacturer) and external quality assessment every month (Hemqual program, Sociedad Española Hematología y Hemoterapia).

**Table I**

Hematologic Parameters (Mean ± SD) in the δβ-Thalassemia Trait, β-Thalassemia Trait, and Iron Deficiency Anemia

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<tr>
<td>RBC, ×10¹²/L</td>
<td>5.84 ± 0.60</td>
<td>NS</td>
<td>5.95 ± 0.68</td>
<td>&lt; .001</td>
<td>4.99 ± 0.59</td>
<td>&lt; .001</td>
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<tr>
<td>Hb, g/dL</td>
<td>12.09 ± 1.22</td>
<td>NS</td>
<td>11.90 ± 1.37</td>
<td>&lt; .001</td>
<td>10.79 ± 1.20</td>
<td>&lt; .001</td>
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| MCV, fl | 66.39 ± 2.35 | < .001 | 64.82 ± 3.43 | < .001 | 31.16 vs 29.34 g/dL, *P* < .001 | Mean MCV (64.82 vs 72.31 fl, *P* < .001), MCH (20.04 vs 21.72 pg, *P* < .001), and RDW (16.04% vs 17.59%, *P* < .001) were lower. Significant differences in the four RBC subpopulations were also found: %MACRO (0.02% vs 0.13%, *P* < .001), %MICRO (34.27% vs 18.41%, *P* < .001), %HYPER (0.69% vs 0.26%, *P* < .001), and %HYPO (21.36% vs 34.18%, *P* < .001). All parameters evaluated showed statistically significant differences between δβ-TT and IDA. RBC count (5.84 ± 10¹²/L vs 4.99 ± 10¹²/L, *P* < .001), Hb levels (12.09 vs 10.79 g/dL, *P* < .001), MCHC (31.16 vs 29.34 g/dL, *P* < .001), and RDW (16.04% vs 17.59%, *P* < .001) were higher in δβ-TT. Mean MCV (66.39 vs 72.31 fl, *P* < .001) and MCH (20.73 vs 21.72 pg, *P* < .001) were higher in IDA. RBC subpopulations analysis revealed higher %MACRO in patients with IDA (0.13% vs 0.03%, *P* < .001), whereas %MICRO was more
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... elevated in those with δβ-TT (32.22% vs 18.41%, P < .001). %HYPER was higher in δβ-TT as well (0.91% vs 0.26%, P < .001), and %HYPO was lower (18.16% vs 34.18%, P < .001). ROC analysis of classic hematologic parameters and RBC subpopulations is summarized in Table 2.

In the second step of the study, only RDW (AUC, 0.914) was evaluated to discriminate between δβ-TT and β-TT, since the AUC of MCH and MCV (0.668 and 0.662, respectively) revealed poor diagnostic performance. When a threshold of 17.35% for RDW was applied in the validation group, 185 cases of β-TT (84.9%) and 37 cases of δβ-TT (90.2%) were accurately identified.

Discussion

The treatment of the two most common causes of microcytic anemia is quite different, so it is crucial to distinguish them properly. The study of microcytosis should always include iron parameters. If iron levels are normal or elevated, a thalassemia should be suspected. An elevation of HbA₂, regardless of HbF values, leads to the diagnosis of β-TT. However, if HbA₂ values are normal and determination of HbF is not performed, the patient cannot be properly diagnosed. Both δβ-TT and α-TT show normal values of HbA₂, with elevated and normal HbF values, respectively. Therefore, determination of HbF values plays a key role in the differential diagnosis of patients with thalassemia and, in our opinion, should always be performed. Otherwise, δβ-TT may be misdiagnosed.

δβ-TT vs β-TT

Patients with thalassemia are characterized by an increase in RBC count as a result of the chronic increase in erythropoiesis with normal or slightly low Hb levels. Decreased hemoglobinization and impaired globin chain synthesis lead to a higher number of divisions in erythroid...
precursors and therefore to microcytosis in these patients. Several studies have evaluated classic hematologic parameters in β-TT and δβ-TT.\textsuperscript{6-10,12,19} RDW was the only parameter that showed significant differences between both types of thalassemia in all cases, being more elevated in δβ-TT. Vayá et al\textsuperscript{19} also reported higher MCV and MCHC in patients with δβ-TT, whereas Fernández Valle et al\textsuperscript{10} also found differences in MCV. Our results confirm that patients with δβ-TT have more elevated RDW (18.79% vs 16.04%, $P < .001$) and are also less microcytic and hypochromic. To our knowledge, this is the first study in which the technology of the Advia 2120i was applied to both β-TT and δβ-TT. In previous reports, the CBC of patients with thalassemia was performed with Coulter analyzers\textsuperscript{7-9} or the H*2 analyzer,\textsuperscript{12} or no mention was made of the technology applied.\textsuperscript{6,10}

For unknown reasons, erythroid precursors show a heterogeneous distribution of HbF that can be demonstrated with the Kleihauer-Betke test.\textsuperscript{20} Analysis of the cellular distribution of HbF by flow cytometry shows heterocellular distribution in δβ-TT, whereas the pattern for HPFH is homocellular.\textsuperscript{21} Neither the Kleihauer-Betke test nor a flow cytometry study was performed in our study. Although differences in RDW between both entities have been reported by many authors,\textsuperscript{6-10} none of the published studies analyzed whether HbF influences RDW. Our results demonstrate that RDW is strongly affected by the percentage and the absolute value of HbF in patients with β-TT and δβ-TT. Therefore, if a patient with laboratory features suggestive of thalassemia shows elevated RDW as well, not only δβ-TT but also β-TT with high levels of HbF should be suspected. In addition, although uncommon, δβ-TT carriers may sometimes have normal or slightly elevated RDW. In our study, three (4.05%) of 74 patients with δβ-TT had an RDW of 16.5% or less with lower mean HbF values (2.46%). HbF is increased in patients with δβ-TT due to an overexpression of γ-globin genes by a mechanism of loss of competence of transcription factors that regulate the expression of β-globin genes.\textsuperscript{22} As a consequence of δ and β gene deletion, those transcription factors interact with the γ-locus promoter zones. Phenotypic expression of HbF depends on the size and location of the deleted sequences. Loss of regulatory regions of the expression of γ-globin genes can also influence HbF synthesis.\textsuperscript{23}

In patients with δβ-TT, the increase in γ chain synthesis may partially compensate for the lack of β-globin chain synthesis. Therefore, hemoglobinization of δβ-TT erythroid precursors may be increased, which would explain their higher MCHC, and they may consequently undergo fewer divisions and have higher MCV than that in patients with β-TT. MCH is derived from precise measurements of RBC count and Hb. Many authors recommend using MCH instead of MCV to screen for thalassemia, since the stability of MCH during storage of blood samples is higher.\textsuperscript{4,24} MCH was higher in patients with δβ-TT than in those with β-TT, which can be explained by their higher hemoglobinization and higher MCV. Further studies with a larger sample size of prospectively selected δβ-TT and β-TT carriers are needed to confirm these differences in MCH and MCHC. ROC analysis confirmed that RDW is the most efficient single parameter to discriminate between both pathologies (AUC, 0.914), followed by MCH (AUC, 0.668) and MCV (AUC, 0.662).
In the second step of the study, we prospectively identified 218 cases of β-TT and 41 cases of δβ-TT. In total, 84.9% cases of β-TT (185 of 218) and 90.2% cases of δβ-TT (37 of 41) were accurately identified when the cutoff of 17.35% for RDW was applied in the validation group. These results confirm the reliability of RDW to discriminate between both entities. The slightly lower proportion of properly identified β-TT is probably due to persistence of HbF in some of these patients, which leads to higher RDW. Therefore, it would be of great interest if β-TT carriers with and without persistence of HbF could be accurately identified with any hematologic parameter. This question should be addressed in future studies with prospectively selected patients with β-TT. Neither MCH nor MCV was considered in this second analysis, since none proved to be efficient enough.

Even though δβ-TT is less microcytic and less hypochromic than β-TT, no differences in the four RBC subpopulations were found among them. Before Bonferroni correction was applied, %HYPER had proved to be significantly higher and %HYPO lower in δβ-TT compared with β-TT. This circumstance indicates the need for an even larger sample of prospectively selected patients with δβ-TT and β-TT to evaluate the role of RBC subpopulations in their differential diagnosis.

β-TT vs IDA

Patients with β-TT usually have higher RBC counts, higher Hb levels and MCHC, and lower MCV and RDW compared with patients with IDA. In our study, differences between β-TT and IDA were found not only in classic parameters but also in RBC subpopulations with high statistical significance (P < .001) in all. These results are similar to those reported previously by Urrechaga et al13,14 and Jiménez et al, although Jiménez et al considered β-TT and δβ-TT together as a single group.

Although MCHC was lower in IDA, MCH was higher in patients with IDA compared with those with β-TT. MCH is influenced not only by hemoglobinization but especially by the volume of RBCs.

The volume/hemoglobin concentration erythrogram provided by the Advia 2120i analyzer shows characteristic patterns for both IDA and thalassemia, so a first-sight differential diagnosis can be easily made.13 In addition, numeric values for RBC subpopulations are provided. As expected, mean %MICRO was much more increased in β-TT (34.27%) than in IDA (18.41%; P < .001), while mean %HYPO showed an opposite trend (21.36% for β-TT and 34.18% for IDA; P < .001). To our knowledge, neither %MACRO nor %HYPER has ever been analyzed in patients with β-TT and IDA. A possible explanation for the more elevated %MACRO in patients with IDA could be that bleeding patients were included in this group and, consequently, their reticulocyte counts may have been higher. Iron plays a key role in DNA synthesis, being part of ribonucleotide reductase, which catalyzes the formation of deoxyribonucleotides. Therefore, iron deficiency leads to fewer divisions of erythroid precursors and to a certain degree of macrocytosis compared with β-TT.

In previous studies, RBC count and %MICRO have demonstrated to be the most efficient single measurements in the differential diagnosis of IDA and β-TT to date. According to our data, discriminant efficiency of MCV (AUC, 0.879) was higher than RBC count (AUC, 0.871) and %MICRO (AUC, 0.840), which ranked second and third, respectively. %MICRO performed slightly worse than expected, although its AUC was good enough to discriminate both pathologies.

δβ-TT vs IDA

Similar to the comparison between β-TT and IDA, all the parameters (including RBC subpopulations) showed significant differences between δβ-TT and IDA. %HYPER proved to be the most efficient parameter, with an AUC of 0.910, better than RBC count (AUC, 0.856) and MCHC (AUC, 0.854). Most RBCs in IDA are hypochromic, whereas the hemoglobinization of erythroid precursors in δβ-TT is enhanced by the increase in γ chain synthesis. These two factors can explain the differences in %HYPER. Although both entities usually present with elevated RDW, inconsistent differences among them have been reported so far. Similar to results reported by Juncá Piera et al,9 higher RDW was found in δβ-TT (18.79% vs 17.59%, P < .001) compared with IDA in our study. However, Miguel Sosa et al9 did not find those differences. The discrepancy between the published studies may be related to the different criteria of selecting patients and the variety of sample sizes among them.

When a patient with microcytic anemia is evaluated, it is important to bear in mind the possibility of δβ-TT, especially in some geographical areas. This entity can sometimes be mistaken for β-TT and IDA, and since the management of these pathologies is quite different, reliable tools to discriminate them are needed. Genetic counseling for δβ-TT carriers is different from that given to β-TT carriers. Patients with homozygous δβ-thalassemia usually have thalassemia intermedia, which means they can often manage a normal life but may need occasional transfusions depending on the severity of their anemia. On the other hand, symptoms of homozygous β-thalassemia major include severe anemia, splenomegaly, bone deformities, and frequent transfusions that may lead to iron overload. Both classic hematologic parameters and RBC subpopulations have confirmed to be useful in selecting microcytic samples for thalassemia testing, and some (RDW) can discriminate efficiently between δβ-TT and β-TT. To our knowledge, this is the first study that evaluates RBC subpopulations in patients with δβ-TT. However, a possible drawback in our study is the fact that molecular analysis was not performed in all samples.

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Sequencing of the β-globin gene was performed only in those samples with inconclusive percentages of HbaA and Hbf.

In conclusion, although RBC parameters obtained from hematologic analyzers are automated and rapid tools for a presumptive diagnosis in patients with microcytic anemia, they cannot replace the iron panel and determination of both HbaA, and Hbf (when thalassemia is suspected). We demonstrated here the value of RDW as a simple diagnostic tool in the hematology laboratory. When a patient with suspected thalassemia has an elevated RDW, the diagnosis is more likely to be δβ-TT, especially with an RDW of 17.35% or higher in our experience. However, the possibility of β-TT with elevated Hbf should also be considered. The RDW is strongly correlated to the percentage of Hbf, such that a patient with high Hbf levels will have more anisocytosis than a patient with normal levels of Hbf. This correlation has not been reported so far. Although RDW was the most reliable parameter to discriminate δβ-TT from β-TT, some others such as MCH, MCV, and MCHC may be also helpful in doubtful cases. Further studies are needed to confirm these results and clarify the efficiency of RBC subpopulations on prospectively selected patients with δβ-TT and β-TT. Finally, both RBC subpopulations and classic hematologic parameters provide good discrimination between thalassemia (δβ-TT and β-TT) and IDA.

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References


