### **Comparison Study of the Eosin-5'-Maleimide Binding Test, Flow Cytometric Osmotic Fragility Test, and Cryohemolysis Test in the Diagnosis of Hereditary Spherocytosis**

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### ABSTRACT

**Objectives:** Current guidelines recommend the eosin-5'maleimide (EMA) binding test and cryohemolysis test for screening for hereditary spherocytosis (HS), and the flow cytometric osmotic fragility (FC OF) test was recently developed to replace the classic OF test. We evaluated the performance of the EMA binding test, FC OF test, cryohemolysis test, and the hemoglobin (Hb)/mean corpuscular hemoglobin concentration (MCHC) ratio in the diagnosis of HS and assessed whether these tests reflect the clinical severity of HS.

*Methods:* A total of 153 patients with anemia (33 with HS, 40 with autoimmune hemolytic anemia, 40 with anemia of chronic disease, and 40 with iron deficiency anemia [IDA]) and 140 healthy controls were enrolled, and the performance of the three tests was evaluated.

**Results:** Both the EMA binding test (area under the curve [AUC], 0.996) and the FC OF test (AUC, 0.992) performed satisfactorily, but the cryohemolysis test (AUC, 0.723) performed significantly worse because of false positivity in patients with IDA. The Hb/MCHC ratio ( $\mathbf{P} < .001$ ) was able to reflect the clinical severity of HS.

**Conclusions:** Our results demonstrate that both the EMA binding and FC OF tests are useful as screening tests for the diagnosis of HS, but the cryohemolysis test has limited use due to its false positivity in IDA, with the Hb/MCHC ratio the most useful parameter for assessing the clinical severity of HS.

Upon completion of this activity you will be able to:

- identify the best test(s) to be applied for the discrimination of the patients with hereditary spherocytosis from those with other anemias.
- identify the most suitable results reporting method applicable to these test(s).

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Hereditary spherocytosis (HS) is the most common form of inherited hemolytic anemia, with an incidence of 1 per 2,000 to 5,000.<sup>1-6</sup> The classic diagnostic methods for HS are based on evaluating the degree of hemolysis induced by a hypotonic solution (the classic osmotic fragility test [OFT]) or glycerol. However, the classic OFT has some pitfalls, such as frequent indeterminate test results and lack of a standardized cutoff value for positivity.<sup>7-12</sup> In 1990, the cryohemolysis test, which detects the greater fragility of RBCs in patients with HS to a temperature shift from 37°C to 0°C, was introduced as a complementary test to the classic OFT, and it was shown to possess superior sensitivity/specificity for diagnosing HS compared with the classic OFT.13 This test was also reported to identify all patients with HS, including asymptomatic carriers, which probably reflects the cryohemolysis test's dependency on the membrane defect, not the surface to volume ratio of RBCs.14

Other HS diagnostic methods include visual examination of RBC membrane proteins by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and measurement of eosin-5'-maleimide (EMA) binding to RBCs by flow cytometry (the EMA binding test). The SDS-PAGE method has some disadvantages, such as a strict requirement for expensive equipment, variable detection sensitivity depending on the nature of the defective protein, and ethnic variation.<sup>15,16</sup> The EMA binding test can yield consistent results with refrigerated samples, and previous studies have reported better performance of the EMA binding test for diagnosing HS compared with the classic OFT.<sup>17-22</sup> However, the decrease in fluorescence intensity of the EMA reagent stored at room temperature<sup>23</sup> and the requirement of a flow cytometer are major obstacles. In addition, the method of reporting the results of the EMA binding test has not been standardized; some laboratories report the results as absolute mean fluorescence intensities (MFIs) of EMA in patient RBCs, whereas others use percentages of normal controls.<sup>8-12,17-21</sup> Recently published guidelines for diagnosing HS recommend both the EMA binding test and the cryohemolysis test for screening.<sup>6</sup>

Recently, the flow cytometric osmotic fragility (FC OF) test, which measures the proportion of residual RBCs after the induction of hemolysis by flow cytometry, has been developed as a complementary method to the classic OFT.<sup>23</sup> This method can quantify the vulnerability of RBCs to hemolysis and generates a precise numerical value representing osmotic fragility. Two studies have evaluated the performance of the FC OF test and reported satisfactory diagnostic sensitivity and specificity for diagnosing HS.<sup>23,24</sup>

Another important issue is the identification of a parameter reflecting clinical severity in patients with HS. Although a reduction in the ratio of hemoglobin (Hb) to mean corpuscular hemoglobin concentration (MCHC) is associated with increased HS clinical severity,<sup>25</sup> the clinical use of HS diagnostic test results for this purpose has not been validated. In addition, most previous studies have compared the performance of the HS diagnostic test with that of the classic OFT based on a comparison between patients with HS and healthy controls. Since anemia of chronic disease (ACD), iron deficiency anemia (IDA), and autoimmune hemolytic anemia (AIHA) are the main types of anemia, studies using these patients as controls are needed. However, to our knowledge, such studies have not been performed.

In this study, we performed three HS diagnostic tests (the EMA binding test, FC OF test, and cryohemolysis test) in 153 patients with anemia (33 with HS, 40 with AIHA, 40 with ACD, and 40 with IDA) and 140 healthy controls. We evaluated and compared the performance of the tests with respect to three issues to identify the most appropriate test for the diagnosis of HS: discrimination of HS patients from patients

with other kinds of anemia, consistency of interpretation of the test results, and ability to reflect clinical severity.

### **Materials and Methods**

### **Patient Selection and Specimen Collection**

A total of 33 patients diagnosed with HS from October 2012 through May 2013 were enrolled in this study. The diagnostic criteria for HS were anemia (<11.5 g/dL for patients aged 2-12 years, <13.0 g/dL for male patients >12 years old, and <12.0 g/dL for female patients >12 years old), hyperbilirubinemia (total bilirubin >1.2 mg/dL), splenomegaly, reticulocytosis ( $\geq$ 1.5% for patients aged 2-6 or 12-15 years,  $\geq$ 1.9% for patients aged 6-12 years, and  $\geq$ 1.8% for patients  $\geq$ 15 years), and spherocytosis in peripheral blood (PB) ( $\geq$ 2/high-power field). The thresholds used were those employed in the authors' institution, and the guidelines for the standardization of PB smear interpretation were from the Korean Society of Laboratory Hematology.<sup>26</sup>

For AIHA, 40 patients with evidence of hemolysis, such as anemia, hyperbilirubinemia, increased serum lactate dehydrogenase (LD) (>250 IU/L), and positivity in the direct antiglobulin test (DAT), were enrolled. For ACD, 40 patients who had a normal or increased serum ferritin level ( $\geq$ 20 ng/mL for male patients and  $\geq$ 10 ng/mL for female patients) and a normal or decreased serum transferrin level ( $\leq$ 360 mg/dL) were enrolled. For IDA, 40 patients with decreased serum ferritin and iron ( $\leq$ 50 µg/dL) and increased total iron binding capacity (TIBC;  $\geq$ 400 µg/dL) were enrolled.

In addition, 140 healthy adults who underwent general health examinations in the authors' institution were selected as controls, and their laboratory data and medical history were reviewed to confirm their status as healthy controls. Residual PB samples obtained at the time of diagnosis or general health examination were used for the EMA binding test, FC OF test, and cryohemolysis test. All clinical and laboratory data were obtained by retrospective review of electronic medical records, in which were recorded the sex, age, Hb levels, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCHC, reticulocyte counts, serum total bilirubin, haptoglobin, LD, iron, TIBC, ferritin, transferrin, plasma Hb, frequency of spherocytes in PB, existence of splenomegaly, strength of DAT, and classic OFT results. This study was approved by the international review board of the authors' institution.

### **EMA Binding Test**

In total, 100  $\mu$ L EDTA blood was added to the tubes with 2 mL phosphate-buffered saline (PBS) and washed three times, and 5  $\mu$ L RBC suspension was added to each tube. Then, 25  $\mu$ L



of 0.5 mg/mL EMA (Sigma-Aldrich Company, Poole, Dorset, UK) stored at -80°C was added to each tube, followed by incubation in the dark for 1 hour at room temperature. After washing three times with 2 mL PBS, 500 µL PBS was added to each tube. The final RBC suspensions from patients and controls were prepared with 100 µL RBC suspension from each tube and 1.4 mL PBS. Flow cytometric analysis was performed with a FACSCanto II (Becton Dickinson, San Jose, CA), and 15,000 RBC events were acquired. After the acquisition of scattergrams for forward scatter (FSC) and side scatter (SSC), RBCs with high FSC and SSC were gated, and MFI values (mean channel fluorescence) for the RBCs from patients and controls were obtained in the FL1 channel. RBCs emit green fluorescence when they bind EMA. Test results were represented as EMA (%), the percentage of the EMA binding values (MFIs) of the patients' RBCs relative to those of the age-matched controls. An example of the interpretation of the EMA binding test results is presented in **Figure 11**. The assay was performed in triplicate for each patient and in duplicate for each control.



**Figure 11** An example of the interpretation of EMA binding test results: mean of triplicates (**A**) or duplicates (**B**) from patient or healthy control. Patient: (373 + 355 + 347)/3 = 358. Healthy control: (452 + 444)/2 = 448. EMA (%): (358/448) × 100 = 79.9%. EMA, eosin-5'-maleimide. FITC, fluorescein isothiocyanate; FSC, forward scatter; mcf, mean channel fluorescence; SSC, side scatter.

#### Flow Cytometric Osmotic Fragility Test

In total, 1.1 mL normal saline was added to a microcentrifuge tube and to a fluorescence-activated cell sorting (FACS) tube. To standardize the number of RBCs per tube, we calculated the blood volume to be added from the following equation: 130/RBC number/10<sup>6</sup> µL. After adding patient blood to the microcentrifuge tube, 1.0 mL normal saline was added and the mixture was vortexed gently. The final patient RBC suspensions were prepared by transferring a 10-µL RBC suspension from the microcentrifuge tube to the paired FACS tube containing 1.1 mL normal saline. Flow cytometric analysis was performed with the FACSCanto II (Becton-Dickinson). RBCs gated on high FSC and SSC were acquired for 10 seconds, and the number of acquired RBCs was defined as R1. After inducing hemolysis by adding 0.9 mL distilled water and gentle vortexing, the acquisition was continued for 2 minutes, and scattergrams of time/SSC plots divided into 10 sections (R2-R11, each for 10 seconds) were obtained. The residual RBC numbers acquired in each section were recorded. The degree of hemolysis was evaluated from



**Figure 21** An example of the interpretation of flow cytometric osmotic fragility test results before inducing hemolysis (**A**) and after inducing hemolysis with the addition of distilled water (**B**). Residual RBC (%) =  $[(1350 + 1264)/2/32,651 \times 100 = 4.00\%]$ . FSC, forward scatter; SSC, side scatter; ####, not calculated.

the percentage of residual RBCs after inducing hemolysis and represented as residual RBCs (%) by using the following formula:  $[(R6 + R7)/2/R1] \times 100$  (%).

Since two previous reports used the residual RBC counts obtained 2 minutes after induction of hemolysis and applied a dilution factor (1.1/2.0),<sup>23,24</sup> the residual RBCs (%) have been calculated from the equation  $[(R10 + R11)/2] \times 100(\%)/(R1 \times 1.1/2.0)$ . In this study, we modified the previously applied method by using R6 and R7 without application of the dilution factor. This modification was performed to improve the reporting method into a simpler one, which

reduces the reporting time. The performance of this new modified reporting method in the discrimination of HS from other anemias was validated in our present study. We found that the modified reporting method demonstrated satisfactory performance (area under the curve [AUC], 0.992), which is similar to the previously used method (AUC, 0.991) for discriminating HS from patients with other kinds of anemia. Therefore, we used the modified reporting method (the use of R6 and R7 without the application of dilution factor) as the proposed reporting method in this study. An example of the interpretation of FC OF test results is given in **Figure 21**.

### **Cryohemolysis Test**

Two tubes containing 2 mL sucrose solution (0.7 mol/L, 21.47 g sucrose dissolved in 100 mL of 50 mmol/L PBS) and two tubes containing 2 mL distilled water were prepared, and all tubes were incubated in a 37°C humidified incubator. Then, 200 µL EDTA blood samples were washed three times with 2 mL cold PBS and stored in an ice box (0°C). Next, 50 µL of patient or normal control RBC suspension (adjusted to hematocrit 50%-70%) stored at 0°C was added to each tube, vortexed for 2 seconds, and incubated for 10 minutes in the 37°C humidified incubator. Then all the tubes were moved to a 0°C ice box and left for 10 minutes. After centrifuging all the tubes, 100  $\mu$ L of each supernatant was added to a fresh tube containing 2 mL distilled water and vortexed for 2 seconds, and the optical density (OD) at 540 nm was read in a spectrophotometer (U-3310; Hitachi, Tokyo, Japan). The extent of cryohemolysis was calculated as follows:

Cryohemolysis (%) = (mean OD values of two tubes containing sucrose/mean OD values of two tubes containing distilled water)  $\times$  100 (%).

### Reference Ranges and Cutoff Values Applicable to Each Test and the Hb/MCHC Ratio

We established the reference ranges and cutoff values for the determination of HS positivity in the three tests and for the Hb/MCHC ratio, according to the Clinical Laboratory Standards Institute guidelines for the determination of reference ranges in clinical laboratory tests.<sup>27</sup> To establish the reference ranges, we used the test results from 140 controls and applied the Kolmogorov-Smirnov test to assess the normality of the distribution of results.

### Performance of Each Test and the Hb/MCHC Ratio for Discriminating HS From Other Anemias

The results of the three HS diagnostic tests and Hb/ MCHC ratios were compared among patients with HS, other patient subgroups, and healthy controls. The sensitivity, specificity, negative predictive value, positive predictive value, and accuracy of each test for discriminating patients with HS from patients with other kinds of anemia were estimated based on the previously determined cutoff values. Subsequently, a receiver operating characteristic (ROC) curve analysis was performed to determine the best cutoff values applicable to each test, and the performances of the tests were compared using the best cutoff values. For this comparison, we used AUC values and the 95% confidence interval calculated for each test. In addition, Spearman correlation analysis was performed to evaluate the correlations between test results.

### Performance of Each Test and the Hb/MCHC Ratio for Assessing the Clinical Severity of HS

Thirty-three patients with HS were classified into three

subgroups (mild HS, moderate HS, and severe HS), and the results of each test and the Hb/MCHC ratio were compared pairwise between the subgroups. The published criteria for HS clinical severity<sup>6</sup> were applied to the present study as follows: Hb 11.0 to 15.0 g/dL, reticulocytes 3% to 6%, or total bilirubin 1.7 to 3.4 mg/dL for mild HS; Hb 8.0 to 11.0 g/dL, reticulocytes 6% to 10%, or total bilirubin 3.4 to 5.1 mg/dL for moderate HS; Hb 6.0 to 8.0 g/dL, reticulocytes more than 10%, or total bilirubin more than 5.1 mg/dL for severe HS. In addition, we compared the test results of the patients with mild HS with those with moderate to severe HS to evaluate the ability of each test to specifically discriminate patients with mild HS.

### **Statistical Analysis**

The Pearson  $\chi^2$  test or Fisher exact test was used to compare differences of dichotomous variables, and the Mann-Whitney *U* test was used to compare continuous variables. ROC curve analysis was used to calculate AUC values for each test to discriminate patients with HS from patients with other kinds of anemia. Correlations between pairs of test results were obtained by Spearman correlation analysis. All tests were two-tailed, with  $P \leq .05$  considered significant. All analyses except for the ROC curve analysis were performed using SPSS 13.0.1 for Windows (SPSS, Chicago, IL), and MedCalc version 9.2.0.2 (MedCalc Software, Ostend, Belgium) was used for the ROC curve analysis.

### Results

## Reference Ranges and Cutoff Values for the Screening Tests and Hb/MCHC Ratio

In the EMA binding test, EMA (%) was normally distributed, and the reference ranges and the cutoff value evaluated in the 120 patients with anemia other than HS were determined to be 86.9% to 118.7% and less than 86.9%, respectively. In the FC OF test, residual RBCs (%) were not normally distributed, and the above values determined for the 140 controls were 6.6% to 71.6% and less than 6.6%, respectively. In the cryohemolysis test, cryohemolysis (%) was not normally distributed, and the above values established in the normal controls were 1.5% to 11.8% and more than 11.8%, respectively. The Hb/MCHC ratio also was not normally distributed, and the above values in the normal controls were 0.36 to 0.48 and less than 0.36, respectively. These results are summarized in **Table 11**.

#### **Classic OFT Results in Patients With HS**

The classic OFT was performed in 26 (78.8%) of the 33 patients. Eighteen (69.2%) patients showed increased OF.

Table 1 Reference Ranges and Cutoff Values Applicable to the EMA Binding Test (EMA [%]), FC OF Test (Residual RBC [%]), Cryohemolysis Test (Cryohemolysis [%]), and Hemoglobin/MCHC Ratio

Test	Distribution: Kolmogorov- Smirnov Test ( <i>P</i> Value)	Reference Range: 2.5th-97.5th Percentile or Mean ± 1.96 SD	Estimated Cutoff Value: 2.5th/97.5th Percentile or Mean ± 1.96 SD	
EMA binding test, EMA (%)	Normal (.083)	86.9-118.7	<86.9 (mean ± 1.96 SD)	
Cryphemolysis test cryphemolysis (%)	Not normal (<.001) Not normal ( 002)	6.6-71.6 1 5-11 8	<6.6 (2.5th percentile)	
Hemoglobin/MCHC ratio	Not normal (<.001)	0.36-0.48	<0.36 (2.5th percentile)	

EMA, eosin-5'-maleimide; FC OF, flow cytometric osmotic fragility; MCHC, mean corpuscular hemoglobin concentration.

Three (11.5%) patients showed increased OF after 24 hours of incubation. Five (19.2%) patients did not show increased OF. Among these five patients, OFT after 24 hours of incubation was not performed in four (15.4%) patients, and only one patient in whom OFT was performed did not show increased OF after 24 hours of incubation. From these results, the diagnostic sensitivity of classic OFT was estimated to be 21 (80.7%) of 26.

### Comparison of Clinical and Laboratory Findings Between Patient Subgroups

The patients with HS had significantly higher Hb levels, MCHC values, and reticulocyte counts than did those with AIHA, ACD, and IDA. They also had lower MCV than did the patients with AIHA and ACD and higher total bilirubin and LD levels than did the patients with ACD and IDA. With regard to the Hb/MCHC ratio, the patients with HS had significantly higher ratios than did those with AIHA, ACD, and IDA but lower ratios than the healthy controls. These differences can be attributed to the following situation: the decrease of Hb and increase of MCHC in patients with HS led to a lower Hb/MCHC ratio than in the healthy controls but a higher ratio than in the patients with AIHA, ACD, and IDA, who have significantly more severe anemia than do patients with HS. These results are described in **Table 21**.

### **Comparison of the Test Results in the Patient Subgroups**

In the EMA binding test, the patients with HS had significantly lower EMA (%) than did those with AIHA, ACD, and IDA. However, there were no significant differences between the other three patient subgroups. In the FC OF test, the patients with HS also had significantly lower residual RBCs (%) than did those with AIHA, ACD, or IDA and the healthy controls. In the cryohemolysis test, the patients with HS had higher cryohemolysis (%) than did those with AIHA or ACD and the healthy controls. However, the patients with IDA exhibited significantly higher cryohemolysis (%) than did those with HS, AIHA, or ACD and the healthy controls. These results are summarized in Table 2 and **Figure 31**.

# Performance of the Tests for Discriminating HS From Other Anemias

When the predefined cutoff values were applied, the performances of both the EMA binding test and FC OF test were satisfactory for discriminating patients with HS from those with other anemias, since the sensitivity, specificity, negative predictive value, positive predictive value, and accuracy were calculated to be 97.0%, 97.5%, 99.2%, 91.4%, and 97.4%, respectively, for the EMA binding test and 93.9%, 97.5%, 98.3%, 91.2%, and 96.7%, respectively, for the FC OF test. However, the corresponding values for the cryohemolysis test were 48.5%, 76.7%, 84.4%, 36.4%, and 70.6%, respectively, markedly lower in terms of sensitivity and PPV than the former two tests. These results are described in **Trable 31**.

Subsequent ROC curve analysis confirmed the superiority of both the EMA binding test and FC OF test over the cryohemolysis test, as the AUC values obtained for the EMA binding test (0.996) and FC OF test (0.992) were significantly better than those obtained for the cryohemolysis test (0.723) (all P < .001). All these results are summarized in **Table 41** and **IFigure 41**.

### Correlations Among the Test Results Obtained From 153 Patients With Anemia

EMA (%) was positively correlated with residual RBCs (%) and negatively correlated with cryohemolysis (%). This means that the interpretations of the EMA binding test results and the FC OF test/cryohemolysis test results are consistent. However, the FC OF test results did not correlate with those of the cryohemolysis test. These results are represented in Table 3.

## Evaluation of the Tests and Hb/MCHC Ratio for Assessing the Clinical Severity of HS

EMA (%), residual RBCs (%), and cryohemolysis (%) did not change significantly with increasing severity of HS. However, the Hb/MCHC ratio was significantly lower in patients with moderate or severe HS compared with those with mild HS, and a trend toward a decreased Hb/MCHC ratio was also observed in patients with severe HS compared with

			<i>P</i> Values						
Variable	HS (1) (n = 33)	AIHA (2) (n = 40)	ACD (3) (n = 40)	IDA (4) (n = 40)	HC (5) (n = 140)	1 vs 2	1 vs 3	1 vs 4	1 vs
Sex, M/F, No. <sup>b</sup>	16/17	22/18	25/15	16/24	77/63	NS			
Age, median (range), $\gamma^c$	8.0 (1.0-64.0)	51.5 (17.0-78.0)	63.0 (17.0-86.0)	45.0 (14.0-75.0)	38.0 (17.0-72.0)	<.001			
EMA binding test, EMA (%), median (range) <sup>c</sup>	71.2 (59.1-90.1)	102.0 (79.6-127.0)	103.7 (87.4-129.0)	100.8 (83.6-124.0)	ND	<.001			NA
FC OF test, residual RBCs (%), median (range) <sup>c</sup>	2.8 (0.4-15.7)	44.6 (4.1-94.3)	36.0 (5.6-105.3)	60.0 (26.9-136.0)	25.1 (3.6-79.0)	<.001			
Cryohemolysis test, cryohemolysis (%), median (range) <sup>c</sup>	11.0 (3.1-54.2)	3.6 (1.0-13.5)	4.2 (1.0-15.8)	16.0 (2.7-66.6)	4.7 (1.1-19.1)	<.001	<.001	.043	<.0
Hemoglobin, median (range), g/dL <sup>c</sup>	11.8 (6.1-16.0)	8.9 (5.0-11.9)	8.4 (5.6-11.1)	8.8 (4.0-12.7)	14.2 (12.0-18.3)	<.001			
MCV, median (range), fL <sup>c</sup>	82.3 (74.9-99.4)	91.3 (73.8-128.3)	90.8 (72.7-116.7)	72.9 (52.6-90.1)	89.0 (77.0-98.0)	<.001			
MCHC, median (range), g/dL <sup>c</sup>	35.6 (31.0-37.9)	34.1 (28.9-37.6)	33.6 (30.4-36.0)	30.1 (22.7-32.6)	34.0 (30.1-37.0)	<.001			
Reticulocyte, median (range), % <sup>c</sup>	6.32 (0.74-13.84)	2.48 (0.11-28.59)	1.58 (0.09-4.60)	1.15 (0.50-3.51)	ND	<.001	NA		
Total bilirubin, median (range), mg/dL <sup>c</sup>	1.7 (0.3-7.8)	1.2 (0.3-12.4)	0.6 (0.2-7.9)	0.4 (0.1-1.3)	ND	NS	<.001	<.001	NA
LD, median (range), IU/L <sup>c</sup>	345.0 (189.0-785.0)	327.5 (145.0-896.4)	226.5 (126.0-483.0)	175.0 (128.0-199.0)	ND	NS	<.001	<.001	NA
Hemoglobin/MCHC ratio, median (range) <sup>c</sup>	0.33 (0.18-0.47)	0.26 (0.16-0.36)	0.25 (0.17-0.33)	0.29 (0.18-0.40)	0.41 (0.05-0.49)	<.001	<.001	.002	<.0
Spherocytosis degree, 0/1+/2+/3+, % <sup>b</sup>	18.2/33.3/ 42.4/6.1	100.0/0.0 /0.0/0.0	100.0/0.0/ 0.0/0.0	100.0/0.0/ 0.0/0.0	NA	<.001			NA
Presence of splenomegaly, % <sup>b</sup>	57.6	0.0	0.0	0.0	NA	<.001			NA
History of splenectomy, $\%^{\rm b}$	30.3	0.0	0.0	0.0	NA	<.001			NA
Strength of DAT (+),	100.0/0.0/0.0/	0.0/35.0/37.5/	100.0/0.0/0.0/	100.0/0.0/0.0/	NA	<.001			NA

Table 2

ACD, anemia of chronic disease; AIHA, autoimmune hemolytic anemia; DAT, direct antiglobulin test; EMA, eosin-5'-maleimide; FC OF, flow cytometric osmotic fragility; HC, healthy controls; HS, hereditary spherocytosis; IDA, iron deficiency anemia; LD, lactate dehydrogenase; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; NA, not applicable; ND, not done; NS, not significant.

0.0/0.0

0.0/0.0

<sup>a</sup> The patients with IDA showed significantly higher cryohemolysis (%) than did those with AIHA or ACD and the healthy controls (all P < .001).

20.0/7.5

<sup>b</sup> P values were calculated using the  $\chi^2$  test.

0/1+/2+/3+/4+, %<sup>b</sup>

<sup>c</sup> P values were calculated using the Mann-Whitney U test.

those with moderate HS. Subsequent comparisons confirmed the decrease in the Hb/MCHC ratio in patients with moderate to severe HS (P < .001) compared with those with mild HS. These results are summarized in **Table 5**.

0.0/0.0/

### Discussion

In the present study, the cutoff value for positivity in the EMA binding test was estimated to be EMA (%) less than 86.9%, which is slightly higher than that of a previous study, which reported a cutoff value of more than a 17.0% decrease

in the mean channel fluorescence value (<83.0%) compared with the controls.<sup>23</sup> In the cryohemolysis test, we determined the cutoff value to be more than 11.8%, which is different from the 2.8%<sup>13</sup> suggested in previous studies. These results imply that it is critical for accurate reporting to determine the reference range and cutoff value applicable to the EMA binding and cryohemolysis tests in each individual laboratory. In the EMA binding test, additional analysis demonstrated the superior performance of EMA (%) compared with EMA (MFI), which uses the mean of triplicate MFI values (mean channel fluorescence) obtained from patients' samples (AUC,

/s 5

.001

.001



0.996 vs 0.798; P < .001) (data not shown). We were thus able to establish the superior performance of EMA (%) over EMA (MFI). Given that the strength of fluorescence by EMA is expected to be decreased in proportion to the reaction time, it can be speculated that the EMA (%), which reflects the relative proportion of fluorescence compared with that in healthy controls, can yield more reliable results than EMA (MFI). In addition, our present results could demonstrate these speculations described above.

In the FC OF test, two previous reports used the residual RBC counts obtained 2 minutes after induction of hemolysis and applied a dilution factor.<sup>23,24</sup> We modified this method by using residual RBC counts detected 1 minute after inducing hemolysis without applying any dilution factor. Additional analysis has demonstrated satisfactory performance of both the previous method (AUC, 0.991) and the present modification (AUC, 0.992) for discriminating HS from other anemias (data not shown). We thus propose a simpler method for the FC OF test, which reduces reporting time and produces results similar to, or better than, the original method.

■Figure 3■ Schematic boxplots representing four test results obtained from each patient subgroup (A, EMA binding test;
B, flow cytometric osmotic fragility test; C, cryohemolysis test; D, hemoglobin/MCHC ratio). A dotted line in each figure indicates each cutoff value for each test: 86.9% for EMA (%), 6.6% for residual RBCs (%), 11.8% for cryohemolysis (%), and 0.36 for hemoglobin/MCHC ratio. ACD, anemia of chronic disease; AIHA, autoimmune hemolytic anemia; EMA, eosin-5'-maleimide; Hb, hemoglobin; HC, healthy control; HS, hereditary spherocytosis; IDA, iron deficiency anemia; MCHC, mean corpuscular hemoglobin concentration. \**P* < .001.</li>

In our study, we obtained significantly lower EMA (%) values in patients with HS than in those with other anemias, but there were no differences in EMA (%) among patients with AIHA, ACD, and IDA. In contrast, residual RBCs (%) decreased in the following order: HS, healthy control, ACD, AIHA, and IDA. These results reflect the utility of the FC OF test for the differential diagnosis of anemia as well as for discriminating HS from other anemias. It is noteworthy

Table 3

No. of Patients								
Test and Result	Not HS	HS	Total	Sensitivity, %	Specificity, %	NPV, %	PPV, %	Accuracy, %
EMA binding test,	EMA (%)							
Negative	117	1	118	07.0	07.5	00.2	01 /	07.4
Positive	3	32	35	97.0	97.5	99.2	91.4	97.4
FC OF test, residu	al RBCs (%)							
Negative	117	2	119	02.0	07 6	00.2	01.0	06.7
Positive	3	31	34	93.9	97.5	90.3	91.2	90.7
Cryohemolysis tes	st, cryohemo	olysis (%)						
Negative	92	17	109	10 E	76 7	011	26.4	70.6
Positive	28	16	44	40.0	70.7	04.4	30.4	70.0
Hemoglobin/MCHC ratio								
Negative	3	12	15	62.6	2.5	20.0	15.0	157
Positive	117	21	138	03.0	2.0	20.0	10.2	10.7

Performance of the Four Test Results for Discriminating Patients With Hereditary Spherocytosis From Those With Other Anemias<sup>a</sup>

EMA, eosin-5'-maleimide; FC OF, flow cytometric osmotic fragility; HS, hereditary spherocytosis; MCHC, mean corpuscular hemoglobin concentration; NPV, negative predictive value; PPV, positive predictive value.

<sup>a</sup> Sensitivity, specificity, negative predictive value, positive predictive value, and accuracy for the diagnosis of hereditary spherocytosis were calculated based on the following cutoff values for each test, as in Table 1: 86.9% for EMA (%), 6.6% for residual RBCs (%), 11.8% for cryohemolysis (%), and 0.36 for hemoglobin/MCHC ratio.

### Table 4 Data From Receiver Operating Characteristic Curve and Correlation Analysis

			Performance When the Best Cutoff Values Are Applied, %		P Values Between Two AUCs/ Spearman Correlation Coefficient (P Value)				
Test	AUC (95% CI)	Best Cutoff	Sensitivity	Specificity	EMA Binding Test	FC OF Test	Cryohemolysis Test	Hemoglobin/ MCHC Ratio	
EMA binding test, EMA (%)	0.996 (0.969- 0.998)	≤85.0%	97.0	98.3	NA	.570/0.442 (<.001)	<.001/-0.373 (<.001)	<.001/-0.311 (<.001)	
FC OF test, residual RBCs (%)	0.992 (0.962-0.999)	≤10.8%	97.0	95.8	.570/0.442 (<.001)	NA	<.001/0.016 (.840)	<.001/-0.261 (.001)	
Cryohemolysis test, cryohemolysis (%)	0.723 (0.645-0.793)	>7.1%	84.8	63.3	<.001/-0.373 (<.001)	<.001/ 0.016 (.840)	NA	.165/0.302 (<.001)	
Hemoglobin/ MCHC ratio	0.813 (0.742-0.871)	>0.28	78.8	74.2	<.001/-0.311 (<.001)	<.001/ -0.261 (.001)	.165/0.302 (<.001)	NA	

AUC, area under the curve; CI, confidence interval; EMA, eosin-5'-maleimide; FC OF, flow cytometric osmotic fragility; MCHC, mean corpuscular hemoglobin concentration; NA, not applicable.

that we found a higher cryohemolysis (%) in patients with IDA than in those with HS, which points to the occurrence of some false positivity among patients with IDA. We have been unable to determine the cause of this phenomenon, which is an important question for the future.

ROC curve analysis confirmed the superior performance of both the EMA binding test and FC OF test compared with the cryohemolysis test, and we can conclude that both of the former are useful for discriminating HS from other anemias, whereas the cryohemolysis test is not useful, which is partly discordant with current guidelines.<sup>6</sup> Correlation analysis underscored this conclusion since the results of the FC OF test were consistent with those of the EMA binding test but not with those of the cryohemolysis test.

As shown in Tables 3 and 4, one patient gave a falsenegative result in the EMA binding test and two gave falsenegative results in the FC OF test. The former patient was definitely positive in both the FC OF test (residual RBCs, 2.1%) and the cryohemolysis test (18.9%). The two latter patients were positive in the EMA binding test (85.0% and 81.9%) but also negative in the cryohemolysis test (4.5% and 7.3%). These three patients possessed definite HS features. In addition, three patients gave false-positive results in the EMA binding test, and the other three patients did in the FC OF



**IFigure 4** Receiver operating characteristic curves of the four test results for discriminating patients with hereditary spherocytosis from those with other anemias. EMA, eosin-5'-maleimide; FC OF, flow cytometric osmotic fragility; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

test. Three patients with false positivity in the EMA binding test were definitely negative in both the FC OF test (residual RBCs, 45.5%, 60.9%, and 64.5%) and cryohemolysis test (6.0%, 3.3%, and 7.0%). Similarly, three patients with false positivity in the FC OF test were definitely negative in both the EMA binding test (109.0%, 98.0%, and 107.0%) and the cryohemolysis test (3.8%, 3.3%, and 5.2%). All six patients did not possess definitive HS features.

However, the results of EMA binding test, FC OF test, and cryohemolysis test did not correlate with the severity of HS. These results were in contrast to the Hb/MCHC ratio, which decreased significantly with increasing clinical severity of the HS. These results are in agreement with previous findings.<sup>25</sup>

The present study has the limitation that the number of patients with severe HS was extremely small (n = 2), so the statistical power of the comparison of test results with respect to the clinical severity of HS was limited. The effect of RBC transfusion on the outcome of the tests in the patients with HS also needs to be considered. Since our patients with HS had mild anemia (median Hb, 11.8 g/dL), only one received an RBC transfusion within 1 week prior to the tests, and the effect of this transfusion on each test was not evaluated adequately. The difference in the FC OF test results between the transfused patient and the patients who did not receive a transfusion (median, 2.8% vs 10.8%) was evident. But the difference in the EMA binding test result (median, 71.2% vs 85.0%) was less evident than that for the FC OF test. However, both tests did not reach statistical significance (P = .121), and we found that the trend of a shift to normal in the transfused patient was detectable in both tests. In addition, when we applied the predefined cutoff value (86.9% in the EMA binding test and 6.6% in the FC OF test), the transfused HS patient in our present study was also positive in the EMA binding test but shifted to negative in the FC OF test. Therefore, it can be speculated that the effect of transfusion (shift to normal) exists in both the EMA binding test and the FC OF test, but the avoidance of the shift by transfusion is more important in the FC OF test than in the EMA binding test to minimize false-negative results. All these observations suggest that an effect of RBC transfusion within 1 week prior to testing may exist in the case of the FC OF test and suggests that the FC OF test should perhaps be avoided in patients who have been transfused within 1 week prior to the test. But it is likely that the avoidance of the shift due to RBC transfusion may be less important in the EMA binding test than in the FC OF test.

#### Table 5

Comparison of the Four Test Results Obtained for the 33 Patients With Hereditary Spherocytosis in Relation to Clinical Severity

	Clinical Severity <sup>a</sup>				P Value <sup>b</sup>				
Test	Mild (n = 26)	Moderate (n = 5)	Severe (n = 2)	Moderate + Severe (n = 7)	Mild vs Moderate	Mild vs Severe	Moderate vs Severe	Mild vs Moderate + Severe	
EMA binding test, EMA (%), median (range)	70.2 (59.1-90.1)	71.5 (68.3-85.0)	75.1 (71.9-78.3)	71.9 (68.3-85.0)	.155	.381	.857	.100	
FC OF test, residual RBCs (%), median (range)	2.8 (0.4-15.7)	4.0 (1.3-10.8)	2.2 (2.2-2.2)	4.0 (1.3-10.8)	.259	.529	.381	.567	
Cryohemolysis test, cryohemolysis (%), median (range)	10.2 (3.1-24.1)	21.4 (4.5-54.2)	12.3 (8.7-15.9)	15.9 (4.5-54.2)	.452	.825	.857	.467	
Hemoglobin/MCHC ratio, median (range)	0.35 (0.29-0.47)	0.26 (0.23-0.28)	0.21 (0.18-0.23)	0.25 (0.18-0.28)	<.001	.005	.095	<.001	

EMA, eosin-5'-maleimide; FC OF, flow cytometric osmotic fragility; MCHC, mean corpuscular hemoglobin concentration.

<sup>a</sup> Clinical severity of hereditary spherocytosis was defined as mild (hemoglobin 11.0-15.0 g/dL, reticulocytes 3%-6%, or total bilirubin 1.7-3.4 mg/dL), moderate (hemoglobin 8.0-11.0 g/dL, reticulocytes 6%-10%, or total bilirubin 3.4-5.1 mg/dL), and severe (hemoglobin 6.0-8.0 g/dL, reticulocytes >10%, or total bilirubin >5.1 mg/dL) according to guidelines for the diagnosis and management of hereditary spherocytosis (2011 update).<sup>6</sup>

<sup>b</sup> P values were calculated using Mann-Whitney U test.

In conclusion, when the purpose of testing is to discriminate patients with HS from patients with other forms of anemia, both the EMA binding test and the FC OF test are satisfactory and can be recommended, whereas the cryohemolysis test is not recommended because of false positivity in patients with IDA. The most appropriate reporting methods for the EMA binding test and FC OF test are EMA (%), which represents the frequency of EMA binding in a patient's sample compared with a healthy control, and residual RBCs (%), which uses the residual RBC count 1 minute after inducing hemolysis without applying any dilution factor. In addition, the Hb/MCHC ratio is the most useful parameter for assessing the clinical severity of HS.

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