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Abnormalities of Erythrocyte Membrane Proteins in Korean Patients with Hereditary Spherocytosis

Hereditary spherocytosis (HS) is a common inherited erythrocyte membrane disorder characterized by chronic hemolytic anemia. Clinical manifestations and biochemical abnormalities of HS are heterogeneous. In this study, we investigated erythrocyte membrane protein defects in 27 Korean HS cases. Utilizing both the Fairbanks system and the Laemmli system, sodium dodecyl sulfate polyacrylamide gel electrophoresis of erythrocyte membrane proteins was performed. Proteins were stained with Coomassie brilliant blue and gels were scanned using a densitometer. We detected spectrin deficiency in 7.4% of cases (2/27), ankyrin deficiency in 29.6% (8/27), combined spectrin and ankyrin deficiency in 3.7% (1/27), band 3 deficiency in 11.1% (3/27) and protein 4.2 deficiency in 14.8% (4/27). Membrane protein deficiencies were not observed in nine cases (33.3%, 9/27). Members of two of seven families tested showed the same protein defects as the proband. Ankyrin deficiency alone and combined with spectrin deficiency accounted for 33.3% of cases (9/27), and they were the most common biochemical defects in Korean HS cases. Protein 4.2 deficiency caused HS more frequently in Koreans than in Caucasians.

Key Words: *Spherocytosis, Hereditary; Erythrocyte Membrane; Electrophoresis, Polyacrylamide Gel; Ankyrins; Band 4.2 Protein*

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INTRODUCTION

Hereditary spherocytosis (HS) is a common inherited hemolytic anemia characterized by spherocytes in peripheral blood and increased osmotic fragility of erythrocytes (1). This disorder is heterogeneous in clinical presentation, inheritance, molecular basis and biochemical phenotype. In a majority of families, HS is dominantly inherited, though some patients show a recessive pattern of inheritance or new mutation (2). The characteristic features of HS include mild anemia, jaundice and splenomegaly, but clinical severity varies highly ranging from asymptomatic to severe life-threatening hemolytic anemia (1).

The primary defects of HS reside in the erythrocyte membrane cytoskeleton, protein components of the membrane (3). Although ankyrin or spectrin deficiency was detected in a majority of HS patients, recent studies have indicated that the primary molecular lesion could also involve band 3 or protein 4.2 (4, 5).

Other than a few reports describing the biochemical causes of HS patients in the United States and Europe (2, 6), and in Asia except for Japan, biochemical abnormalities of HS patients have not been well documented (7). The purpose of our study is to investigate the distribution of erythrocyte membrane protein defects in Korean HS patients. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), we analysed erythrocyte membrane protein abnormalities in Korean HS patients.

MATERIALS AND METHODS

Materials

We studied 27 HS cases. HS was hematologically diagnosed by clinical features, the presence of spherocytes in peripheral blood smear, increased osmotic fragility, exclusion of other cause of spherocytic hemolytic anemia

and the family history of hemolysis.

Methods

Using heparin as an anticoagulant, venous blood was collected and immediately processed. Erythrocyte ghosts were prepared from blood by hypotonic lysis, using lysing buffer [5 mM $NaPO_4$, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), pH 8.0] according to the procedure of Dodge et al. (8).

Protein concentration was measured by Lowry's method (9), using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). SDS-PAGE was performed utilizing both the methods of Fairbanks (10) and Laemmli (11). For the former, 3.5-17% exponential gradient gel was used with Tris-acetate buffer (pH 7.4), and for the latter, 4-17% linear gradient gel with Tris-HCl buffer (pH 8.8). Proteins were stained with Coomassie brilliant blue, and gels were scanned using a densitometer (KEN EN Tec., Copenhagen, Denmark). The amount of each protein was expressed as a ratio to that of band 3 except for the amount of protein 4.2 which was compared with that of protein 4.1. A protein component deficiency was identified when in the same gel, the amount of each protein in a patient was 10% less than that in a normal control.

RESULTS

Using the Fairbanks system, we observed nine bands in SDS-PAGE gel; these were α and β spectrin, ankyrin, band 3, protein 4.1 and 4.2, actin, protein 6 and protein 7 (Fig. 1A). The Laemmli system did not differentiate the ankyrin band from β spectrin, so eight bands were identified (Fig. 1B). Slower-moving fractions such as spectrin, ankyrin and band 3 were separated better with the Fairbanks system than with Laemmli's, and faster-moving fractions such as protein 4.1 and 4.2, actin, protein 6 and protein 7 were better separated with the Laemmli system. The defects of slower-moving and faster-moving protein components in the Fairbanks and Laemmli systems were analysed.

Hematological data and results of red cell membrane protein studies of HS cases are summarized in Table 1. In seven of 27 HS cases, we analysed red cell membrane proteins of family members and for some, we obtained clinical and hematological data. In other cases, clinical information and blood samples of family members were, unfortunately, not available. Hemoglobin levels ranged from 2.6 to 15.0 g/dL, and all cases showed spherocytosis in peripheral blood smear. Osmotic fragility was increased in 20 cases, normal in one and not evaluated in six.

We detected spectrin deficiency in 7.4% of cases

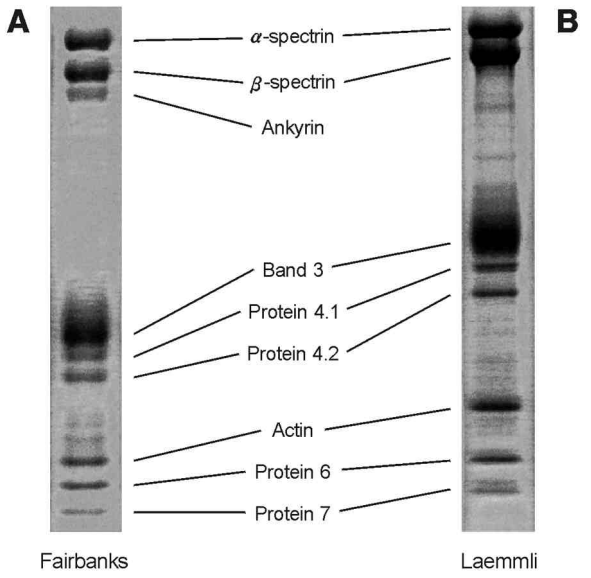

Fig. 1. Erythrocyte membrane proteins in SDS-PAGE. SDS-PAGE utilized both the Fairbanks system with 3.5-17% exponential gradient gel **(A)**, and the Laemmli system with 4-17% linear gradient gel **(B)**; proteins were stained with Coomassie brilliant blue.

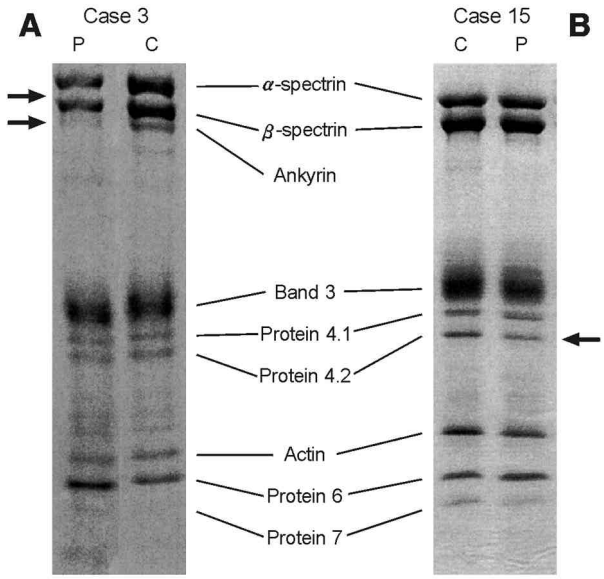

Fig. 2. SDS-PAGE stained by Coomassie brilliant blue of two hereditary spherocytosis (HS) cases. **(A)** Combined ankyrin and spectrin deficiency in HS using Fairbanks system (Case 3). The ratio of ankyrin/band 3 and spectrin/band 3 were 0.11 and 1.00, respectively (normal control: 0.28 and 1.14). **(B)** Partial protein 4.2 deficiency in HS, using Laemmli system (Case 15). The ratio of protein 4.2/protein 4.1 was 0.88 (normal control 1.35). C, normal control; P, patient

(2/27), ankyrin deficiency in 29.6% (8/27), combined spectrin and ankyrin deficiency in 3.7% (1/27) (Fig. 2A), band 3 deficiency in 11.1% (3/27) and protein 4.2 defi-

Table 1. Hematological data and results of erythrocyte membrane protein studies of patients with hereditary spherocytosis and their family members

Case	Age/Sex	Hb (g/dL)	Osmotic fragility	PBS and other findings	Results of SDS-PAGE [The ratio* of patients (normal controls)]
Case 1	3/M	8.0	↑	Spherocytosis	Ank def [ank/b3 0.15 (0.28)]
Case 2	3/M	6.4	↑	Spherocytosis	Ank def [ank/b3 0.18 (0.22)]
Case 3	6/M	6.8	↑	Spherocytosis	Ank & sp def [ank/b3, sp/b3 0.11, 1.00 (0.28, 1.14)]
Brother	4/M	10.1	NA	Spherocytosis	Ank & sp def [ank/b3, sp/b3 0.12, 0.88]
Father		NA	NA	Normal	Normal
Mother		NA	NA	Splenectomy	Ank & sp def [ank/b3, sp/b3 0.16, 0.85]
Case 4	8/F	10.1	↑	Spherocytosis	B3 def [sp/b3 1.51 (1.02)]
Case 5	15/F	2.6	↑	Spherocytosis†	Ank def [ank/b3 0.19 (0.27)]
Brother	16/M	NA	NA	Normal	Normal
Mother	46/F	11.4	↑	Cholecystectomy	Ank def [ank/b3 0.17]
Case 6	11/M	13.8	↑	Spherocytosis	Normal
Mother		NA	NA	NA	Normal
Case 7	5/F	10.4	↑	Spherocytosis	Ank def [ank/b3 0.17 (0.25)]
Sister	3/F	9.7	NA	Spherocytosis	Normal
Case 8	12/M	12.2	↑	Spherocytosis	Ank def [ank/b3 0.16 (0.19)]
Case 9	6/M	7.8	NA	Spherocytosis	B3 def [sp/b3 1.27 (1.14)]
Mother		NA	NA	NA	Normal
Case 10	2/F	10.0	↑	Spherocytosis	Normal
Case 11	14/F	15.0	↑	Spherocytosis	Sp def [sp/b3 0.85 (1.04)]
Case 12	9/M	13.6	↑	Spherocytosis†	Ank def [ank/b3 0.11 (0.18)]
Mother		NA	NA	NA	Normal
Case 13	8/M	7.5	NA	Spherocytosis	Ank def [ank/b3 0.18 (0.27)]
Mother		NA	NA	Normal	Normal
Case 14		NA	NA	Spherocytosis	Normal
Case 15	7/F	NA	NA	Spherocytosis	P4.2 def [p4.2/p4.1 0.88 (1.35)]
Case 16	3/M	8.3	NA	Spherocytosis	P4.2 def [p4.2/p4.1 0.94 (1.96)]
Case 17	8/F	8.8	NA	Spherocytosis	Normal
Case 18	6/F	9.6	↑	Spherocytosis	B3 def [sp/b3 1.22 (1.01)]
Case 19	6/M	8.1	Normal	Spherocytosis	Normal
Case 20	18/M	8.5	↑	Spherocytosis	P4.2 def [p4.2/p4.1 1.00 (1.40)]
Case 21	18/F	10.8	↑	Spherocytosis	P4.2 def [p4.2/p4.1 0.18 (1.31)]
Case 22	10/F	8.7	↑	Spherocytosis†	Sp def [sp/b3 1.07 (1.43)]
Case 23	6/F	1.8	↑	Spherocytosis	Normal
Case 24	5/M	8.7	↑	Spherocytosis	Normal
Case 25	3/F	8.7	↑	Spherocytosis	Normal
Case 26	27/F	10.8	↑	Spherocytosis	Normal
Case 27	1 mo/M	8.7	↑	Spherocytosis	Ank def [ank/b3 0.2 (0.25)]

*The Fairbanks system was used to detect abnormalities of spectrin, ankyrin, band 3 and the Laemmli system for protein 4.2.; †Case with splenectomy

PBS, peripheral blood smear; def, deficiency; Ank, ankyrin; Sp, spectrin; NA, not available; B3, band 3; P4.2, protein 4.2

ciency in 14.8% (4/27)(Fig. 2B). Ankyrin deficiency alone and combined with spectrin deficiency accounted for 33.3% of cases (9/27), and they were the most common defects. In nine HS patients (33.3%), erythrocyte membrane protein defects were not observed.

Members of two of seven families tested showed the same protein defects as the proband (Table 1).

DISCUSSION

Erythrocyte membrane consists of lipid bilayer, cytoskeletal protein and glycoprotein, and the maintenance of its shape depends on the proteins composing of the inner membrane cytoskeleton. Molecular defects in cytoskeletal protein cause hemolytic anemias, and they are categorized as hereditary spherocytosis (HS), hereditary elliptocytosis, hereditary pyropoikilocytosis, or Southeast Asian ovalostomatocytosis (3). HS is the most common erythrocyte membrane disorder.

In terms of clinical manifestation, molecular defect and biochemical phenotype, HS is a heterogeneous disorder (1). Until the 1980s spectrin deficiency was the principal defect reported in HS (12), but several additional types

of membrane protein defects have recently been described. Until recently, ankyrin deficiency alone or combined with spectrin deficiency was detected in a majority of HS patients but defects of other proteins such as band 3 and protein 4.2 have also been reported to cause HS (2, 4, 5, 13-15).

In this study, we investigated the distribution of erythrocyte membrane protein defects in Korean HS patients. Ankyrin deficiency was most common, observed in 9 of 27 cases (33.3%); there were eight ankyrin deficiencies alone and one ankyrin deficiency combined with spectrin deficiency. Among Caucasians with HS, ankyrin deficiency is the most common biochemical abnormality. Delaunay et al. reported that 70% of HS stems from ankyrin mutations (16). Savvides et al. (14) and Pekrun et al. (15) reported that combined ankyrin and spectrin deficiency was detected in most cases of HS; ankyrin defect was considered the primary molecular defect, and spectrin deficiency was secondary to the loss of ankyrin attachment site. In Italian HS patients, spectrin defects account for 43.2% of cases, and ankyrin deficiency alone or combined with spectrin deficiency, 19.7% (2). Among Korean HS patients, however, spectrin defect alone was found in only 7.4% of cases, which was less common than among Caucasians.

Four cases showed partial protein 4.2 deficiency, which was a higher frequency than that reported in other regions (2, 13). In Japan, protein 4.2 deficiency is also a common cause of erythrocyte membrane disorder and is reported to cause spherocytosis or stomatocytosis (17). All cases with protein 4.2 deficiency in this study showed spherocytosis only.

We could not detect any membrane protein defects in nine HS patients (33.3%). Miraglia del Giudice et al. reported that protein defeciencies were not detected in five kindred out of 39 kindred from Italia (2). In Brazilian HS, 30% of cases showed no abnormalities of membrane proteins (13). Jarolim et al. reported that 15% of autosomal dominant HS had no quantitative abnormalities of erythrocyte membrane proteins (18).

Two of seven cases for which family blood samples and clinical and hematological data were available showed the same defects in family members; one with combined ankyrin and spectrin deficiency (Case 3) and one with ankyrin deficiency alone (Case 5). The inheritance pattern of these cases was considered to be autosomal dominant.

Membrane protein deficiencies were also seen in three patients after splenectomy; this procedure can correct hemolytic anemia and decrease spherocytes in peripheral blood, but it does not seem to change red cell membrane protein abnormalities.

We used SDS-PAGE for the analysis of erythrocyte membrane protein abnormalities. SDS-PAGE separates each protein component and allows the quantitation of each band density. SDS-PAGE method is relatively simple and does not need specific instruments, but the results may be affected by factors such as protein loading dose or gel status, and this makes it difficult to compare the band intensity of patients with that of normal controls. The problem can be overcome, however, by determining the ratio of each band density to band 3 or the ratio of protein 4.2 to protein 4.1 and comparing this ratio to that of normal control (13, 17).

In conclusion, this study showed that ankyrin deficiency is the most common biochemical finding in Korean HS patients, and protein 4.2 deficiency is a more frequent cause of HS in both Koreans and Japanese than in Caucasians.

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REFERENCES

1. Palek J, Jarolim P. *Hereditary spherocytosis, elliptocytosis, and related disorders. In: Beutler E, Lichtman MA, Coller BS, Kipps TJ, eds. Williams' Textbook of Haematology, 5th ed. New York: McGraw-Hill Inc., 1995; 536-63.*
2. Miraglia del Giudice E, Iolascon A, Pinto L, Nobili B, Perrotta S. *Erythrocyte membrane protein alterations underlying clinical heterogeneity in hereditary spherocytosis. Br J Haematol 1994; 88: 52-5.*
3. Bens EJ. *The erythrocyte membrane and cytoskeleton: structure, function, and disorders. In: Stamatoyannopoulos G, Nienhuis AW, Majerus PW, Varmus H, eds. The molecular basis of blood diseases. Philadelphia: WB Saunders, 1994; 257-92.*
4. Reinhart WH, Wyss EJ, Arnold D, Ott P. *Hereditary spherocytosis associated with protein band 3 defect in a Swiss kindred. Br J Haematol 1994; 86: 147-55.*
5. Ideguchi H, Nishimura J, Nawata H, Hamasaki N. *A genetic defect of erythrocyte band 4.2 protein associated with hereditary spherocytosis. Br J Haematol 1990; 74: 347-53.*
6. Jarolim P, Brabec V, Ballas SK, Prchal JT. *Biochemical heterogeneity of the hereditary spherocytosis syndrome. Br J Haematol 1992; 81(suppl 1): 35.*
7. Yawata Y, Kanzaki A, Inoue T, Ata K, Wada H, Okamoto N, Higo I, Yawata A, Sugihawa T, Yamada O. *Red cell membrane disorders in the Japanese population: clinical, biochemical, electron microscopic, and genetic studies. Int J Hematol 1994; 60: 23-38.*
8. Dodge JT, Mitchell C, Hanahan DJ. *The preparation and*

chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Byophys 1963; 100: 119-30.

9. Lowry OH, Rosebrough NJ, Farr AL, Randall J. *Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.*
10. Fairbanks G, Steck TL, Wallach DFH. *Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 1971; 10: 2606-17.*
11. Laemmli UK. *Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-5.*
12. Agre P, Casella JF, Zinkham WH, McMillan C, Bennett V. *Partial deficiency of erythrocyte spectrin in hereditary spherocytosis. Nature 1985; 314: 380-3.*
13. Saad STO, Costa FF, Vicentim DL, Salles TSI, Pranke PHL. *Red cell membrane protein abnormalities in hereditary spherocytosis in Brazil. Br J Haematol 1994; 88: 295-9.*
14. Savvides P, Shalev O, John KM, Lux SE. *Combined spectrin and ankyrin deficiency is common in autosomal dominant hereditary spherocytosis. Blood 1993; 82: 2953-60.*
15. Pekrun A, Eber SW, Kuhlmey A, Schroter W. *Combined ankyrin and spectrin deficiency in hereditary spherocytosis. Ann Haematol 1993; 67: 89-93.*
16. Delaunay J. *Genetic disorders of the red cell membranes. FEBS Letters 1995; 369: 34-7.*
17. Yawata Y. *Band 4.2 Abnormalities in Human Red Cells. Am J Med Sci 1994; 307: 190-203.*
18. Jarolim P, Murray JL, Rubin HL, Taylor WM, Prchal JT, Ballas SK, Snyder LM, Chrobak L, Melrose WD, Brabec V, Palek J. *Characterization of 13 novel band 3 gene defects in hereditary spherocytosis with band 3 deficiency. Blood 1996; 88: 4366-74.*