Erythrocyte Membrane Protein Band 3 Predicts Interferon Ribavirin-Induced Anemia

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Abstract

Aim & Background: It was proposed that the differences in erythrocyte membrane protein contents—especially band 4—take a role in the serious anemia related to interferon plus ribavirin (I/R). The aim of this study is to evaluate whether the erythrocyte membrane protein contents predict anemia related to I/R or not. Methods: 180 mcg interferon α 2a once a week and weight adjusted ribavirin daily were given for 48 weeks to fifty patients with chronic hepatitis C. It was diagnosed as anemia when haemoglobin concentration was <10 mg/dL. In the beginning, the erythrocyte membrane protein contents of all patients were separated by “Sodium Dodecyl Sulphate Polyacrylamide Gel Disc Electrophoresis (SDS-PAGE)” and haemoglobin concentrations were measured. Results: Anemia developed in 17 patients (34%). Levels of erythrocyte membrane proteins were as; spectrin: 20.468 ± 2.5902, ankyrin: 4.576 ± 1.2706, B3: 19.240 ± 2.8358, B4.1: 5.628 ± 1.8832, and B4.2: 5.848 ± 1.8030. When the relation between the development of anemia and erythrocyte membrane proteins was investigated, a relation was only found at B3 which was not statistically significant (p = 0.058). When ROC analysis was performed, 95% CI p = 0.035 for B3 (0.517 - 0.792) was found. In patients whose B3 level was below 17.7%, the sensitivity of anemia development risk was calculated as 64.7% and the specificity thereof was calculated as 66.7%. Erythrocyte membrane protein contents by gender were only different at B3 (p = 0.042). Anemia developed in 17 patients (34%). 14 of these patients were of female and 3 were of male gender; the gender played a significant role in terms of anemia (p = 0.003). Conclusions: Erythrocyte membrane protein B3 is not only useful in predicting the patient under the risk of developing anemia, but also it may be useful in preventing it and it may explain why women inclined to anemia.
Keywords
Anemia, Erythrocyte Membrane Protein, Interferon, Ribavirin

1. Introduction
The current standard of care for the treatment of chronic hepatitis C is peginterferon and ribavirin, which can induce a sustained virological response (SVR) in the majority of patients treated [1] [2].

Ribavirin is a purine nucleoside analogue [3]. Even though its mechanism of action is still controversial, it widely prevents the replication of DNA and RNA viruses through the inhibition of inosine monophosphate dehydrogenase, an essential enzyme in the synthesis of guanosine triphosphate [3]. The final step of this chain is the fatal mutagenesis of the RNA genome [3]. Currently, the treatment of patients with hepatitis C consists of interferon-α and ribavirin combination therapy [4] [5]. Combination therapy, in comparison to single-agent interferon treatment, leads to an increase in side effects [4]-[11]. Dose reductions are common and they are essentially required in 10% of patients under combination therapy who develop anemia [3] [4] [5] [6] [7]. The major toxicity related to ribavirin use is haemolytic anemia. This side effect has been associated with the accumulation of ribavirin triphosphate in red blood cells (RBCs), leading to the inhibition of erythrocyte functions [12] [13].

The single most common adverse event and the primary reason for dose modifying ribavirin is haemolytic anemia [1] [2] [14] [15]. The median declines in haemoglobin during the course of the treatment with PEGIFN and RVN is 2.5 g [1] [2] [14] [15]. Approximately 20% of patients have a decline in haemoglobin of 4 g or more [16].

Aim of this study is to investigate whether erythrocyte membrane proteins (EMP) predict interferon ribavirin-induced anemia.

2. Methods

Patient Selection: A total of 50 consecutive prospectively enrolled patients with chronic HCV infection who underwent liver biopsy at Mersin University Faculty of Medicine Hospital comprised the study cohort between January 2007-December 2008. The study protocol was approved by the Human Ethics Committee of our hospitals and all subjects gave written informed consent to participate in the present study. All subjects had antibodies against HCV (Abbot, Axsym. Hepatitis C virus encoded antigen) and detectable HCV RNA by PCR (Amplicor HCV; Roche Diagnostics. Branchburg, NJ).

A complete clinical evaluation was performed on each patient. Baseline characteristics collected at the time of liver biopsy included the age, height, weight and body mass index (BMI). The formula for BMI is weight in kilograms divided by height in meters squared. Information regarding the average current daily al-
cohol intake (g/day) in the past 6 months and past alcohol intake (g/day) before the last 6 months was noted. The patients with diabetes mellitus, heart disease, renal failure, anemia and haemoglobinopathy were excluded.

**Laboratory Test:** In the morning of the liver biopsy, venous blood was drawn after a 12 hour overnight fast to determine the serum levels of alanine aminotransferase, albumin, bilirubin, complete blood count, international normalized ratio, and EMP content. On the 4 week, blood samples were collected in order to measure EMP content and ribavirin levels. The following laboratory values were measured at baseline, and then at weeks 2, 4, 8, 12, 16, 20, 24 and 48 of treatment: haemoglobin, white blood cells (WBCs), neutrophils, platelets and ALT. All biochemical tests were performed using a conventional automated analyzer within the Department of Clinical Chemistry at Mersin University Faculty of Medicine Hospital.

The plasma level of ribavirin at therapy week 4 was measured by high pressure liquid chromatography (HPLC).

**Erythrocyte Membrane Protein Extraction:** The blood samples collected to determine the EMP were centrifuged for 20 min/1000g followed by the removal of the plasma + platelet + leukocyte layer on top. Equal amounts of isotonic phosphate (5 mM NaH2PO4 and 147 mM NaCl) tampon were placed on the underlying cells. It was again centrifuged for 20 min/1000g followed by the removal of the liquid on top. The washing process was repeated three times. Each time, some portion of the upper-lying cells was removed to obtain a pure erythrocyte suspension. What were obtained in the end were light pink erythrocyte membrane proteins remnants, which were stored at −80˚C until EMP determination.

To denature the proteins, 2 ml of electrophoresis pre-treatment solution (1% Sodium Dodecyle Sulphate, 7% Sucrose, 10 mM Tris-HCl (pH: 8)) were added on the erythrocyte membrane protein remnants, and it was stored at 37˚C for 60 minutes.

The denatured samples were electrophoresed with SDS-PAGE. The protein fractions were stained by soaking in 1% of Coomassie Brilliant Blue R-250 overnight at room temperature. Fractions were obtained during the electrophoresis of the membrane proteins extracted from all blood samples collected from patients. These fractions were: Spectrin (Sp), Ankyrin (Ank), Protein Band 3 (B3), Protein Band 4.1 (B4.1), Protein Band 4.2 (B4.2). The quantitative evaluation of the fractions was made with a Densitometer (Beckman Appraise).

**Statistical Analysis:** Once all data was entered into the MedCalc 9.4.2.1 package programme, informative statistics for all parameters were given for both the group with anemia and the group without anemia. Cut-off values were determined according to the values with anemia and without anemia, and also the statistics were calculated. Also the areas below the ROC curve were calculated as per the parameters, and they were compared with the areas which were below the ROC curve as per the other parameters, and the related graphics were drawn.
3. Results

Characteristics of Patients

180 mcg interferon α 2a once a week and weight adjusted ribavirin (Subjects with body weight < 65 kg: 800 mg/day, subjects with body weight < 85 kg > 65 kg: 1000 mg/day, subjects with body weight > 85 kg: 1200 mg/day) daily were given for 48 weeks to fifty patients with chronic hepatitis C (See Table 1). The dose of ribavirin was reduced by 200 mg in patients whose haemoglobin concentrations fell below 10 g/dL during the course of treatment and it was discontinued if the concentration of Hb fell below 8.5 g/dL. There were 25 female and 25 male patients with a median age of 54.86 ± 8.00 years. The patients’ erythrocyte membrane protein levels were as follows: Sp: 20.468 ± 2.5902, Ank: 4.576 ± 1.2706, B3: 19.240 ± 2.8358, B4.1: 5.628 ± 1.8832 and B4.2: 5.848 ± 1.8030. Anemia developed in 17 patients (34%). 14 of these patients were of female and 3 were of male gender; the gender played a significant role in terms of anemia (p = 0.003). Erythrocyte membrane protein contents by gender were only different at B3 (p = 0.042) (See Table 2). Ribavirin treatment was discontinued in 9 (10.58%).

With respect to anemia; the plasma levels of ribavirin was not different (1.384 ± 0.644 mg/ml vs 1.276 ± 0.858 mg/ml; p = 0.390)

When the relation between the development of anemia and erythrocyte membrane proteins was investigated, a relation was only found at B3, which was not statistically significant (p = 0.058). No statistically significant differences were observed in the areas below the curve when activities of EMPs’ in diagnosing anemia were analyzed by pairwise comparison of ROC curve. The calculated p values are as follows: p = 0.210 for SP and B3, p = 0.467 for Sp and Ank, p =

Table 1. The characteristics of patients.

| Parameter                  | Study Group | Non-anemia | Anemia     | p value |
|----------------------------|-------------|------------|------------|---------|
| N (%)                      | 50          | 33 (66%)   | 17 (34%)   | 0.499   |
| Age ± SD (year)            | 54.86 ± 8.00| 54.30 ± 7.53| 55.94 ± 8.97| 0.499   |
| Sex (F/M)                  | 25/25       | 11/22      | 14/3       | 0.003   |
| Hb ± SD (gr/dl)            | 13.728 ± 1.53| 14.352 ± 1.27| 12.518 ± 1.28| <0.001  |
| Htc ± SD (%)               | 40.332 ± 4.01| 41.767 ± 3.68| 37.547 ± 3.10| <0.001  |
| Ferritin ± SD (ng/ml)      | 206.94 ± 325.67| 234.91 ± 376.05| 152.65 ± 192.98| 0.403   |
| Vitamin B12 ± SD (mg/dl)   | 413.400 ± 235.51| 381.758 ± 173.07| 474.824 ± 322.30| 0.188   |
| Folic acid ± SD (mg/dl)    | 9.468 ± 2.69 | 9.533 ± 2.61 | 9.341 ± 2.92 | 0.814   |
| Spectrin ± SD (%)          | 20.468 ± 2.5902| 19.600 ± 2.85 | 19.653 ± 3.26 | 0.953   |
| Ankyrin ± SD (%)           | 4.576 ± 1.2706| 4.570 ± 1.57 | 4.053 ± 1.20 | 0.242   |
| Protein Band 3 ± SD (%)    | 19.240 ± 2.8358| 19.358 ± 2.50 | 17.924 ± 2.40 | 0.058   |
| Protein Band 4.1 ± SD (%)  | 5.628 ± 1.8832| 5.339 ± 1.86 | 5.547 ± 2.11 | 0.723   |
| Protein Band 4.2 ± SD (%)  | 5.848 ± 1.8030| 5.703 ± 1.36 | 6.076 ± 1.70 | 0.404   |
| Ribavirin level (mg/ml)    | 1.324 ± 0.758 | 1.276 ± 0.858 | 1.384 ± 0.644 | 0.390   |


Table 2. Comparison of erythrocyte membrane contents by gender.

| Sex   | Male            | Female           | p value |
|-------|-----------------|------------------|---------|
| n = 50|                 |                  |         |
|       | 25              | 25               |         |
| Spectrin ± SD | 19.500 ± 2.8912 | 19.736 ± 3.0990 | 0.782  |
| Ankyrin ± SD  | 4.364 ± 1.6735  | 4.224 ± 1.2634   | 0.887  |
| Protein Band 3 ± SD | 19.596 ± 2.9054 | 18.144 ± 1.9197 | 0.042  |
| Protein Band 4.1 ± SD | 5.196 ± 1.7558  | 5.624 ± 2.1111   | 0.440  |
| Protein Band 4.2 ± SD | 5.780 ± 1.5853  | 5.880 ± 1.4036   | 0.814  |

B3 level was significantly different between male and female.

0.941 for Sp and B4.1, p = 0.834 for Sp and B4.2, p = 0.552 for B3 and Ank, p = 0.164 for B3 and B4.1, p = 0.301 for B3 and B4.2, p = 0.484 for Ank and B4.1, p = 0.621 for Ank and B4.2. p = 0.788 for B4.1 and B4.2 (Figure 1).

When ROC analysis was performed, 95% CI p = 0.035 for B3 (0.517 - 0.792) was found. In patients whose B3 level was below 17.7%, the sensitivity of anemia development risk was calculated as 64.7% and the specificity thereof was calculated as 66.7% (Figure 2(a) & Figure 2(b)).

4. Discussion

We have observed a relation between the development of anemia and B3 level. The result for B3 in ROC analysis was 95% CI (0.517 - 0.792) p = 0.035. In patients whose B3 level was below 17.7%, the sensitivity of anemia development risk was calculated as 64.7% and the specificity thereof was calculated as 66.7%. We have observed more anemia cases on females (14/17, p = 0.003), the reason of which we have explained with lower levels of B3 compared to males (18.144 ± 1.9197 versus 19.596 ± 2.9054. p = 0.042).

The average maximum reduction in haemoglobin levels was 3.1 g/dL with ribavirin plus conventional interferon, and 3.7 g/dL with ribavirin plus pegylated interferon [2] [17]. Ribavirin causes various degrees of erythrocyte haemolysis in nearly all patients, and necessitates a dose reduction in 7% - 9% of patients under combination therapy [2] [3] [17] [18]. The prevalence of anemia in our study was 34% (17/50). The higher frequency compared to that reported in the literature may be attributable to our definition of anemia as haemoglobin levels below 10 g/dl. Ribavirin treatment was discontinued in 9 (10.58%); these results are consistent with those reported in the literature.

Little is known about the mechanism of anemia developing during IFN/ribavirin combination therapy for HCV infection. Ribavirin causes dose-dependent and reversible haemolytic anemia. Once inside the red blood cells, ribavirin undergoes phosphorylation to its active form that causes depletion of adenosine triphosphate, and ribavirin triphosphate, which interrupts cellular functions, accumulates inside the erythrocytes [19] [20]. The accumulation of ribavirin triphosphate inside the RBCs disrupts antioxidant mechanisms on the erythrocyte
**Figure 1.** ROC analysis of EMP. When the EMPs'; Sp and B3’s (p = 0.210), Sp and Ank’s (p = 0.467), Sp and B4.1’s (p = 0.941), Sp and B4.2’s (p = 0.834), B3 and Ank’s (p = 0.552), B3 and B4.1’s (p = 0.164), B3 and B4.2’s (p = 0.301), Ank and B4.1’s (p = 0.484), Ank and B4.2’s (p = 0.621), B4.1 and B4.2’s (p = 0.788) activities of diagnosing anemia are analyzed by comparison of ROC curve, no statistical differences were observed in the areas below the curve.

**Figure 2.** (a) & (b) ROC analysis of B3. In ROC analysis, 95% CI p = 0.035 for B3 (0.517 - 0.792) was found. In patients whose B3 level was below 17.7%, the sensitivity of anemia development risk was calculated as 64.7% and the specificity thereof was calculated as 66.7%.
membrane and causes oxidative damage, which leads to the removal of the damaged erythrocytes by the reticuloendothelial system [20]. When erythrocytes are exposed to in-vitro ribavirin, the osmotic fragility and deformability of red blood cells are maintained [21].

Homma et al. [22] established a significant correlation between the female gender, advanced age (>60 years) and weight-adjusted ribavirin dosage (>12 mg/kg) and anemia. Erythrocyte ribavirin level was another risk factor that was reported [23]. The only factor that posed a risk for the development of anemia in another our study was age [24]. The risk of anemia increased 1.112-fold with age.

Furthermore, in-vitro studies have shown that RBCs of patients who develop haemolytic anemia during HCV treatment are more susceptible to oxidative stress [25]. Marked differences have been ascertained in the oxidative stress markers and membrane proteins of patients with or without a history of ribavirin-induced anemia [25]. These findings suggest that there exist risk factors related to erythrocytes for ribavirin-induced anemia. It has been suggested that the augmented susceptibility of erythrocytes to oxidative stress and modifications in the erythrocyte membrane content may be a result of alterations in the Adenosylmethionine/Adenosylhomocysteine ratio inside erythrocytes that are caused by ribavirin; however this has not been investigated [26]. It has been shown that total cholesterol, total phospholipids and the cholesterol/phospholipid ratio distinctly increase in the erythrocyte membrane lipid contents in patients receiving IFN/ribavirin treatment [27]. It is particularly contended that these changes in the erythrocyte membrane lipids lead to a decrease in erythrocyte flexibility and membrane viscosity, and may result in the haemolytic anemia related to IFN/ribavirin treatment [27].

The red blood cell (RBC), an anucleated cell, presents a very limited biosynthesis capacity and poor repair mechanisms. Thus, whenever exposed to physical and/or chemical stress. RBC suffers and accumulates physical and/or molecular damage. Several enzymes are involved in the RBC antioxidant defence, such as catalase (Cat), superoxide dismutase (SOD) and glutathione peroxidase (GPx), which is a selenium (Se)-dependent enzyme. The levels of reduced glutathione (GSH) in the cell are also important in protecting the cell from the deleterious action of reactive oxygen species. The ageing of the cell is associated with a decrease in the activity of several enzymes (such as glucose-6-phosphate dehydrogenase [28] [29] and glutathione peroxidase (GPX) [29] and with modifications in membrane proteins [30], which ultimately may lead to its destruction. Modifications in the erythrocyte membrane band 3 protein, by proteolytic cleavage, clustering or exposure of unusual epitopes, trigger the binding of specific anti-band 3 autoantibodies and complement activation, marking the cell for death [31] [32] [33] [34]. Pronounced differences in markers of oxidative stress and membrane proteins exist between patients with and without a history of ribavirin-induced anemia [25]. Patients with previous severe ribavirin-induced anemia had lower levels of protein sulfhydryls and thioredoxin, higher levels of pro-
tein-mixed disulfides and glutathione peroxidase, and a membrane protein pattern consistent with band 4 dimer disaggregation [25]. In vitro studies have shown that erythrocytes of patients who have had haemolysis during treatment of HCV are more susceptible to oxidative stress [25].

De Franceschi L et al. [15] have reported that ribavirin-treated patients showed an increase in aggregated band 3, which was associated with a significantly increased binding of autologous antibodies and complement C3 fragments indicating an erythrophagocytic removal by reticuloendothelial system. Older and damaged RBCs presented higher band 3 aggregation and lower fragmentation [28]. The younger RBCs showed reduced aggregation and higher fragmentation [28].

Human pregnancy is associated with increased oxidative stress [35]. These features may account for some erythrocyte membrane damage in pregnancy [36] [37]. The data of study which investigated erythrocyte band 3 profile as a cumulative marker of oxidative and/or proteolytic damage have suggested band 3 profile as a marker of erythrocyte changes in pregnancy, which are independent of the “physiological anemia” of pregnancy [38].

The development of cellular defects earlier during the RBC life-span leads to their premature removal from the circulation [31] [39] [40]. The pathway for the removal of senescent or damaged RBCs must involve a change in components that are already present in the circulating RBC, since de novo protein synthesis will have terminated before the RBC reaches maturity. In fact, a senescent cell antigen immunologically related to band 3 protein [32] [33] [41], the transmembrane protein known as the anion channel, marks the RBC for death by triggering the binding of a specific autoanti-band 3 antibody, and complement activation [31] [35] [39] [42]. The modified antigenicity of band 3 may result from proteolytic cleavage, clustering or even by exposure of unusual epitopes [39] [43] [44]. Therefore it seems reasonable to assume that any RBC under oxidative and/or proteolytic stress will be marked for death by band 3 modification, and that the band 3 profile may provide a useful marker of biochemical distress. Changes in band 3 observed with aging include a decrease in efficiency in anion transport, in spite of an increase in the number of anion bindings sites, decrease in glucose transport, binding of antibodies to aged band 3, increase in band 3 degradation to smaller fragments, and in situ binding of physiological IgG autoantibodies resulting in cellular removal [45].

Band 3 isoforms. members of the anion exchange family of proteins, are in a number of physiological activities such as cell volume and osmotic haemostasis. HCO\textsubscript{3}/Cl\textsuperscript{−} Exchange, red cell aging, IgG binding and cellular removal, and the maintenance of the structural integrity of cells [45]. The findings of previous studies have confirmed that band 3 is important in acid base haemostasis, O\textsubscript{2}/CO\textsubscript{2} transport, and membrane stability and skeletal organization [45]. Band 3 deficient red cells were very unstable suggesting that the bilayer of the normal red cell membrane may be stabilized by interaction with band 3 [45].
Hence, deficiency in the level of B3 can be so extended that, remaining amount can be unable to preclude oxidative stress caused by ribavirin. Flexibility of erythrocyte membrane can diminish; transformation to senescent erythrocyte form can be accelerated and eventually moved off the circulation. Especially, a decrease in the RBC deformability and membrane fluidity by changes in these RBC membrane proteins was supposed and it is suggested that those changes may result in haemolytic anemia by interferon plus ribavirin treatment.

As a result of this study, we showed that the level of erythrocyte membrane protein band 3 may predict ribavirin induced anemia. There are a lot of band 3 mutations and polymorphisms [46]. Study of naturally occurring band 3 mutations or measuring of the level of band 3 or band 3 profiles may help in the understanding or prediction of the interferon ribavirin induced anemia.

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