

Abnormal increased re-released Hb from RBCs of an intrahepatic bile duct carcinoma patient was detected by electrophoresis release test¹

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Abstract. In this paper, the hemoglobin (Hb) re-released from red blood cells (RBCs) and whole blood of 7 carcinoma patients were studied by using electrophoresis release test (ERT), which was established by our lab. Among the 7 carcinoma patients, the re-released Hb was distinctively increased from an intrahepatic bile duct carcinoma patient during one-dimension isotonic ERT. Different from the others, the result of double-dimension Hb re-release of this intrahepatic bile duct carcinoma patient showed that not only HbA but also HbA₂ could be re-released from both RBCs and whole blood. The result of isotonic & hypotonic ERT which was performed at room temperature showed that more Hb could be re-released from both RBCs and whole blood of the intrahepatic bile duct carcinoma patient than that of the normal control. After keeping the samples at 37°C for 1 hour, the re-released Hb from RBCs could still be found more than that of the normal control, but was disappeared completely from the whole blood sample. To our surprise, when the isotonic & hypotonic ERT was repeated 2 days later at 37°C, the re-released Hb from RBCs of the intrahepatic bile duct carcinoma patient was increased only in tube 4-6, and disappeared in the other tube. Further mechanism research work cannot be continued because of the patient's leave, but ERT is speculated to be a useful and effective technology to observe the physiological or pathological change of RBCs, blood or body in the future.

Keywords: Hemoglobin, red blood cell, electrophoresis release test, intrahepatic bile duct carcinoma

1. Introduction

Electrophoresis release test (ERT) has been established by our laboratory [1, 2]. During ERT, live red blood cells (RBCs) are added directly onto the starch-agarose mixed gel and the electric current perforates the membrane instantaneously. Discontinuous power supply during electrophoresis is the most important innovation of the ERT technique. As to the mechanism of ERT, we primarily speculate that the electric pulse from turning-on and -off the power supply could create plasmatorrhexis of

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RBCs. Hb, existing free in the cytoplasm, would be released during the first cycle of electrophoresis; while the other Hb, binding with the RBC membrane, would be released during the subsequent cycle of electrophoresis [3]. The amount of re-released Hb may be associated with the status of both Hb and RBC membrane, which can be affected by many diseases, such as hematonosis, tumor, diabetes and so on [4-8]. Generally, little re-released Hb can be observed during ERT. Our previous experiments proved that the re-released Hbs were increased distinctively from RBCs of β -thalassemia, diabetic and some general surgical patients [3]. As we known, oxidative damage is the important factor to destroy the RBC membrane [9] and membrane-bound Hb is an important marker of oxidative injury in RBCs [10]. The genesis of tumor is closely related to the oxidative damage of radicals [11-13], but whether it will affect the amount of re-released Hb has not been studied. In this study, the amount of re-released Hb from some upper gastrointestinal cancer patients will be observed.

2. Materials and methods

2.1. Specimens

Our research was approved by the Ethics Committee of Baotou Medical College. Blood samples were collected from the intervention department of the seventh hospital of Baotou. Before the blood samples were collected, all the people who took part in this experiment were asked to sign the consent information. Blood samples were anti-coagulated with heparin and stored at 4°C. Hematology examinations including blood routine, liver function and renal function were performed by the clinical laboratory of the seventh hospital of Baotou.

2.2. Preparation of the RBC suspension and starch–agarose mixed gel

The anti-coagulated blood was firstly centrifuged at 3000 rpm for 10 minutes to isolate RBCs from the plasma, and then wash the RBCs with saline 4 to 5 times until the supernatant was clear. RBCs were then used to perform electrophoresis after 1:1 dilution with saline. Hemolysate was prepared by continuously adding 200 μ L saline and 100 μ L CCl_4 to the RBCs. After turbulent mixing, the sample was centrifuged at 12 000 rpm for 10 minutes and the upper red hemolysate was pipetted out carefully and stored at 4°C for later use [1-3]. The 2% starch–agarose mixed gel (4:1) was prepared with TEB buffer (pH 8.6) as described previously [1].

2.3. One-direction ERT

5 μ L of RBC suspension and whole blood were added on the starch–agarose mixed gel. The electrophoresis was ran at 6 V/cm for 15 minutes, then paused for 15 minutes and ran for another 15 minutes by turns, and the total electrophoresis time was about 2 hours. After electrophoresis, the red bands on the gel were firstly observed with eyes and then sequentially stained with Ponceau Red and Benzidine [1].

2.4. Double-direction ERT

First, 5 μ L of RBC suspension (about 1.5×10^9 RBCs) and whole blood were added on the starch–agarose mixed gel and one-direction ERT was performed as described above. Then change the

direction of electric field, which is vertical to the original direction. Each directional electrophoresis was ran for 15 min, and then paused for 15 min by turns, and the total electrophoresis time was about 4 hours.

2.5. Isotonic & hypotonic ERT

The electrophoresis method was the same as one-direction ERT, but the RBC suspension and whole blood needed to be diluted with H₂O in the proportion from 10: 0 to 1: 9 (named as tube 1 to 10 respectively), and then kept them at room temperature or 37°C for 1 hour.

3. Results

3.1. One-direction ERT of blood samples from 7 upper gastrointestinal carcinoma

In this experiment, the re-released Hb from 7 upper gastrointestinal carcinoma patients were detected by one-direction ERT. As shown in Figure 1, the main electrophoretic bands are albumin (exists in whole blood, but not in RBCs), HbA and HbA₂. After the first cycle of electrophoresis, there was some red sediment stayed at the origin. Then during each cycle of the “run-pause-run” electrophoresis, there was other Hb re-released from the sediments. Among these 7 patients, patient 1 and 7 were cardia carcinoma, patient 2, 3, 4, 5 and 6 were hepatocellular carcinoma. The re-released Hb ladder of patient 3, 5, 6 and 7 were increased, but the increase of patient 3 (intrahepatic bile duct carcinoma) was especially distinctive.

3.2. Double-direction ERT and isotonic & hypotonic ERT were performed with the blood of patient 3

Double-direction HRT result (Figure 2A) showed that not only HbA but also HbA₂ could be re-released from the origin of the intrahepatic bile duct carcinoma patient. The result of isotonic & hypotonic ERT at room temperature (Figure 2B) showed that more Hbs could be re-released from both whole blood and RBCs samples of intrahepatic bile duct carcinoma patient than the normal control. After keeping the samples at 37°C for 1 hour (Figure 2C), the re-released Hbs from RBCs could still been found more than that of normal control, but the re-released Hb ladder from whole blood sample was disappeared completely. In addition, the red albumin bands stained by Ponceau Red

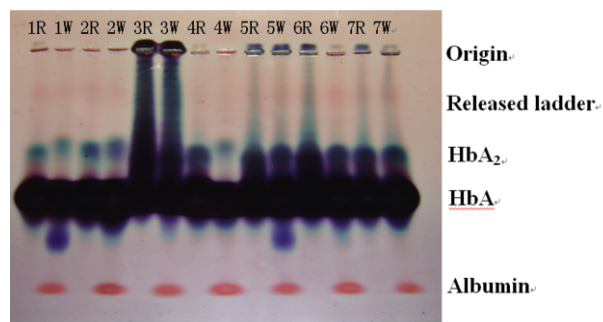


Fig. 1. One-direction ERT of 7 upper gastrointestinal carcinoma blood samples. There were 7 samples; each sample was divided into whole blood group (W) and RBC group (R) correspondingly. Patient 1 and 7 were cardia carcinoma, patient 2, 3, 4, 5 and 6 were hepatocellular carcinoma.

were found to be stained blue slightly by Benzidine. The most interesting result appeared 2 days later, when the isotonic & hypotonic ERT was repeated at 37°C, the released Hb ladder from tube 4-6 of RBC samples were found to be increased, but those from the other tubes were disappeared completely. Also, in the whole blood sample, the red albumin bands stained by Ponceau Red were stained blue by Benzidine distinctively.

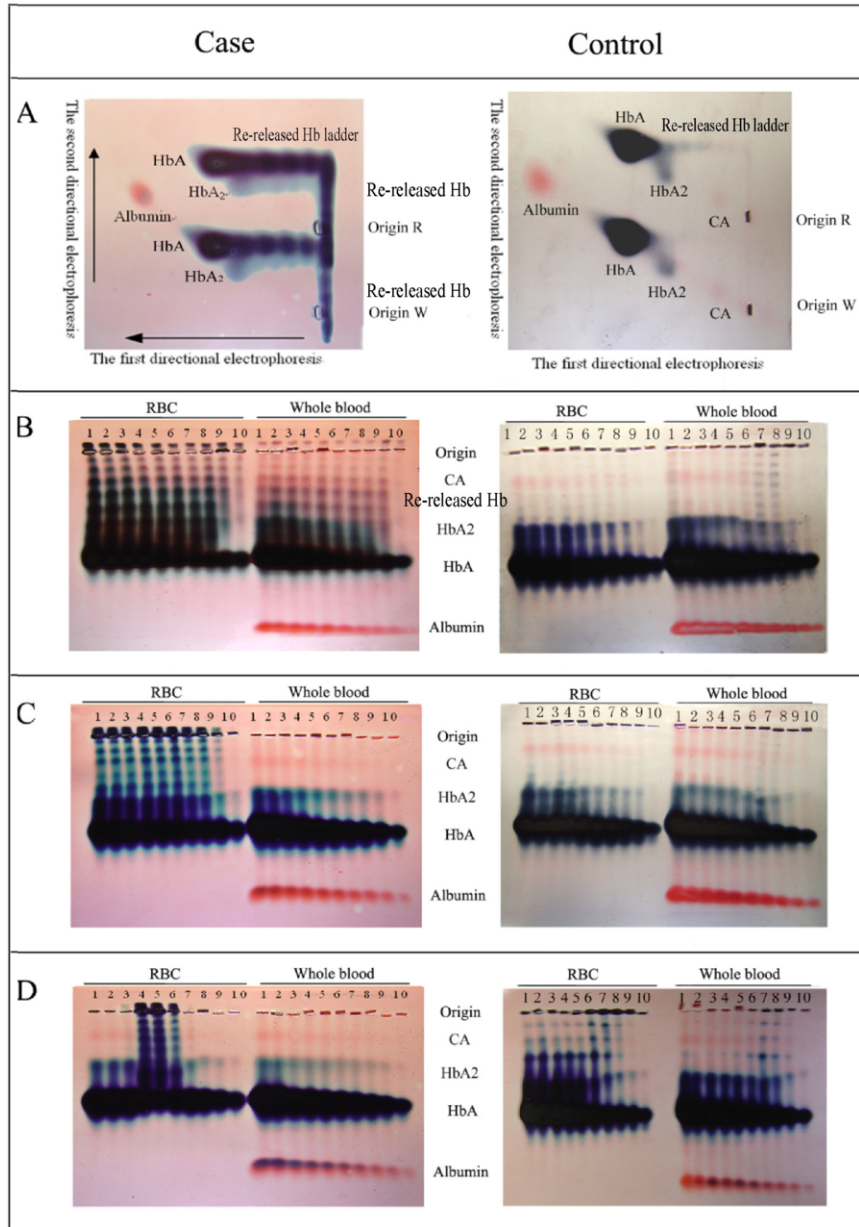


Fig. 2. Different kind of ERTs of the intrahepatic bile duct carcinoma patient. (A) Double-direction ERT; (B) Isotonic & hypotonic ERT at room temperature; (C) Isotonic & hypotonic ERT at 37°C; (D) Isotonic & hypotonic ERT at 37°C two days later.

4. Discussion

ERT is a new method established by our laboratory to study the membrane binding Hb of RBC. During experiments, this technology had been continuously optimized and some new ERT methods were developed, such as double-direction ERT and isotonic & hypotonic ERT. Double-direction ERT could help us to observe the re-released HbA₂, which usually cannot be observed easily during one-direction ERT. Isotonic & hypotonic ERT could help us observe the resistance of RBC membrane to the change of osmotic pressure. In this experiment, the red Hb bands can be observed directly without any staining. In order to observe the trace hemoglobin band better and distinguish hemoglobin band with the other protein, the gel was sequentially stained with Ponceau Red and Benzidine after ERT. Ponceau Red can stain all the protein red (including Hb), but Benzidine can specifically stain Hb blue. So where the blue band exists, there must have hemoglobin. In our results, the blue bands are Hb, and the two main red bands are albumin (fast moving) and carbonic anhydrase (slow moving) respectively. Hemolysis of RBCs leads to Hb leakage, so the albumin bands of whole blood samples can be double stained by Ponceau Red and Benzidine in Figure 2D.

The increased Hb re-release was firstly observed in β -thalassemia patient [1], and then it was observed in diabetes patients and some general surgical patients [3]. To the contrary, the re-released Hb could also decrease distinctively or disappear from RBCs of hereditary spherocytosis patient. We have proved that the membrane integrity and oxidative damage could affect the amount of re-released Hb. Lose of RBC membrane leads to the decrease of re-released Hb, but the oxidative damage can increase the amount of re-released Hb. In this study, not only HbA but also HbA₂ could be re-released from the intrahepatic bile duct carcinoma patient distinctively, and this phenomenon had not been observed in any other patients as yet. In the past, HbA₂ was also speculated to be re-released from RBCs, but it was difficult to be observed due to its relative small amount. As to this case, the re-released HbA₂ was increased distinctively. During isotonic & hypotonic ERT, the re-released Hb ladder of the intrahepatic bile duct carcinoma was also increased distinctively than normal control not only at room temperature but also at 37°C. However, the most interesting phenomenon was that some re-released Hb bands were disappeared from the isotonic & hypotonic ERT (Figure 2D). We speculate that the membrane structure of RBCs might be destroyed after keeping the blood at room temperature for 2 days, but why the increased Hbs only disappeared in tube 1-3 and 7-10 could not be explained up to now. When we were going to do some further research, the patient had leaved the hospital because of economic difficulties. We could not continue our research, but this case report makes our mind to clarify the mechanism and clinical application of ERT in the future.

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