Preliminary investigation about the expression of tubulin in platelets from patients with iron deficiency anemia and thrombocytosis

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**Objective:** In order to inquire into the pathogenesis of increased platelet counts in peripheral blood of patients with iron deficiency anemia (IDA), the phenomenon of thrombocytosis was confirmed, and then the expression of tubulin within platelets from IDA patients was investigated.

**Methods:** Peripheral blood samples were collected from 79 patients with IDA and were divided into 2 groups, group of IDA with normal platelet counts (34 cases), and group of IDA with increased platelet counts (thrombocytosis) (45 cases). Additionally, 45 peripheral blood samples from healthy volunteers were enrolled as a group of healthy controls. Count of platelets in peripheral blood was detected by means of LH-780 hematology analyzer and hemocytometer under a microscope respectively, and analyzed statistically.

**Results:** There was no statistical difference between platelet counts detected by LH-780 hematology analyzer and hemocytometer under a microscope between the latter two groups ($P > .05$). The mean fluorescence intensity (MFI) of both $\alpha$-tubulin and $\beta$-tubulin within platelets from IDA patients with thrombocytosis was significantly less than that from healthy volunteers and IDA patients with normal platelet counts ($P < .01$), and there was no statistical difference between the latter two groups ($P > .05$).

**Conclusion:** Some patients with IDA are accompanied by thrombocytosis, from which the expression of $\alpha$-tubulin and $\beta$-tubulin within platelets reduced obviously compared with those with normal platelet counts and healthy controls respectively. It is implied that downregulation of tubulin probably is a part of the pathogenesis leading to increased platelet counts in IDA.

1. Introduction

Iron deficiency anemia (IDA) is microcytic hypochromic anemia caused by various iron deficiency. In clinical work, we find that some patients suffer from IDA are accompanied by increased platelet counts in peripheral blood (thrombocytosis), which have been being reported in domestic and overseas literatures [1,2]. While, the concrete reason of thrombocytosis occurred on patients with IDA is unclear up to now. Some domestic scholars believe that thrombocytosis in IDA patients is a kind of pseudo-thrombocytosis caused by a mistake of hematology analyzer, namely, some micro-erythrocytes are judged as platelets by hematology analyzer, because of their small volumes. More scholars, however, believe that the increased platelet counts in peripheral blood of IDA patients is authentically due to enhanced thrombopoiesis.

Cytoskeleton composed of microtubules which are formed by the polymerization of a dimer of alpha and beta tubulin is the main component in platelets structure, which plays an important role in the process of thrombopoiesis. While the dominant basis of microtubule is tubulin, a kind of spherical molecules, which contain three types, alpha tubulin, beta tubulin and gamma tubulin. Alpha tubulin and beta tubulin display a similar three-dimensional structure, by which they are tightly combined into a dimer, and subsequently assemble into a microtubule.

As tubulin participates in the formation of platelets and their activation, whether thrombocytosis occurred in IDA is related to tubulin or not? In the present research, we detected platelet count in the peripheral blood of IDA patients by means of LH-780 hematology analyzer and hemocytometer under a microscope.
respectively, after excluding that the increased platelet count was not due to the mistake of LH-780 hematology analyzer, which took micro-erythrocytes and their fragments as platelets, we observed emphatically the expression of tubulin within platelets from IDA patients accompanied by thrombocytosis, so as to investigate the probable pathogenesis of thrombocytosis occurred on IDA patients and therefore offer a caution for the differential diagnosis of IDA and possibly thrombosis (Tables 1 and 2).

2. Materials and methods

2.1. Materials

2.1.1. Patients enrollment

Seventy-nine cases of patients with IDA were enrolled (26 males and 53 females), aged from 19 to 48 years old (the average age was 33.87 years old), who were diagnosed with IDA in the department of hematology of the Second Affiliated Hospital of Shantou University medical college from October 2015 to December 2016. Research objects inclusion criteria: (1) older than 18 years old, if female patients, they must be without pregnancy; (3) Hb < 120 g/L (adult male), Hb < 110 g/L (adult female); (3) erythrocyte mean corpuscular volume (MCV) < 80 fl, mean corpuscular hemoglobin (MCH) < 27 pg, erythrocyte mean corpuscular hemoglobin concentration (MCHC) < 320 g/L; (4) serum iron (Fe) < 9 μmol/L, serum ferritin < 12 mg/L; (5) excluding other metabolic diseases, inflammation, wasting disease and other diseases which may lead to increased platelets [3,4].

According to the count of platelets (platelet counts ≥300 × 10^9/L) determined by LH-780 hematology analyzer, 79 blood samples from IDA patients were divided into 2 groups, group of IDA with normal platelet counts (34 cases, 11 cases of male and 23 cases of female, aged from 22 to 47 years old), and group of IDA with increased platelet counts (45 cases, 15 cases of male and 30 cases of female, aged from 19 to 48 years old).

As healthy controls, 45 cases of healthy volunteers in simultaneous period, who were without anemia (14 cases of male and 31 cases of female, aged from 19 to 49 years old) were enrolled, from Physical Examination Center of Second Affiliated Hospital of Shantou university medical college. They have eliminated the infection, tumor, severe liver and kidney disease and connective tissue disease. Furthermore, iron-related drugs were surely not taken within at least 1 month.

The study protocols were conducted according to the principles of the Declaration of Helsinki, and were approved by the Scientific and Medical Ethical Committee of the Second Affiliated Hospital of Shantou University Medical College. All the subjects gave their written informed consent (appended at the last page) before their inclusion in the study.

3. Methods

3.1. Specimen collection

Fasting peripheral venous blood samples were collected from 79 patients with IDA and 45 healthy controls, and then anticoagulated with ethylene diamine tetraacetic acid-potassium 2 (EDTA-K2) (final concentration 1.5 mg/ml).

3.2. Detection of blood by LH780 hematology analyzer

All peripheral blood samples were examined by LH-780 hematology analyzer for count of White Blood Cell, Red Blood Cell, Hemoglobin, Red blood cell specific volume, MCV, MC and MCHC.

### Table 1. Main reagents.

| Names of reagents | Manufacturers |
|-------------------|---------------|
| Goat Anti-Rabbit IgG + H | MULTI SCIENCES Co., Ltd., HangZhou, China |
| FITC | |
| Anti-alpha Tubulin antibody ab18251 | Abcam plc. (Cam. UK) |
| Anti-beta Tubulin antibody ab13568 | Abcam plc. (Cam. UK) |
| Mouse monoclonal anti-human CD61-PE | Beckman Coulter (CA, U.S.A.) Co., Ltd. |
| Sheath fluid | Beckman Coulter (CA, U.S.A.) Co., Ltd. |
| Cleaner | Beckman Coulter (CA, U.S.A.) Co., Ltd. |
| IntraPrep Reagent 1: Fixation | Beckman Coulter (CA, U.S.A.) Co., Ltd. |
| IntraPrep Reagent 1: Permeabilization | Beckman Coulter (CA, U.S.A.) Co., Ltd. |
| PBS | OuMeng Medical Diagnostics Co., Ltd., Beijing, China |
| Ammonium oxalate | LangFang Peng Cai Fine Chemical Co., Ltd. LangFang, China |
| EDTA-Na2 | Zhengzhou New and Suitable Chemical Products Co., Ltd., ZhengZhou, China |

### Table 2. Main instruments and apparatuses.

| Instruments | Manufacturers |
|-------------|---------------|
| Hemocytometer | Shanghai Biochemical Reagent Refinement Instrument Co., Ltd., ShangHai, China |
| EPICSXL-MCL flow cytometry | Beckman Coulter Co., Ltd., CA, U.S.A. |
| EXPO32 ADC analysis software | Beckman Coulter Co., Ltd., CA, U.S.A. |
| Desktop centrifuge | Biofuge Prime R Co., Ltd., U.S.A. |
| Vertical refrigerator | Hualing Technology Group Co., Ltd., GuangZhou, China |
| Vortex oscillator | HuaiDa Experimental Equipment Co., Ltd., TaiChang, China |
| EDTA-K2 vacuum blood collection tube | Hubel Jinjing Technology Development Co., Ltd., WuHan, China |
| pH meter | CORNING Co., Ltd., New York, U.S.A. |
| 500 ml Measuring cylinder | Beckman Coulter Co., Ltd., CA, U.S.A. |
| 0.1–2 μl Pipettes | Eppendorf Co., Ltd., Hamburg, Germany |
| 20–200 μl Pipettes | Finnpipette Co., Ltd., Finland |
| 100–1000 μl Pipettes | Finnpipette Co., Ltd., Finland |
| Electronic balance | Sartorius Co., Ltd., Hamburg, Germany |
| LH780 hematology analyzer | Beckman Coulter Co., Ltd., CA, U.S.A. |
| DXC800 Automatic Biochemical Analyzer | Beckman Coulter Co., Ltd., CA, U.S.A. |
3.3. Detection of serum iron (Fe) and ferritin

All peripheral blood samples were examined by DXC800 Automatic Biochemical Analyzer for serum iron (Fe) and ferritin.

3.4. Count of platelets under a microscope

3.4.1. Methods

(1) Preparation of ammonium oxalate diluent: 1.0 g of ammonium oxalate and 0.012 g of EDTA-Na2 were weighted on an electronic balance then placed in an Erlenmeyer flask (200 ml). The next, distilled water was added into the flask to 100 ml. Then it was shaken until it was completely dissolved and filtered to clarify the diluent.

(2) 0.38 ml of ammonium oxalate dilution was added into 20 μl of EDTA-K2 anticoagulant venous blood (ammonium oxalate 80 mM, EDTA-Na2 3.2 mM), and mixed well then allowed to stand for 10 min.

(3) A small amount of the mixture was dropped into the counting plate, then the coverslip was covered and allowed to stand at room temperature for 10 minutes. The counting area was filled with the mixture with capillary action. The number of platelets in five small squares in the central squares was counted under a microscope (Figures 1 and 2).

3.4. Count

Count of platelets \((N \times 10^9/L)\) = The number of platelets in five small squares in the central squares \((N) \times 5 \times 10 \times 20 \times 10^5/L\)

Figure 1. Picture of hemocytometer.

Figure 2. Schematic diagram of counting principle on grid line (cells on the line were counted according to the principle of 'Count the cells above without counting the cells below, and count the left cells without counting the right').

3.5. Detection the expression of tubulin in platelets with flow cytometry

3.5.1. Isolation of platelets

The collected venous blood samples were centrifuged at 200 g for 20 min at 25°C. Platelets rich plasma (PRP) was separated and 400 μl of PRP was injected into the flow tube with pipettes and 2 ml of phosphate buffered saline (PBS) was added into the flow tube, then the samples in flow tubes were centrifuged at 1000 g for 10 min. The next, supernatant was discarded to get the sediment. Sediment in the tube was mixed well with 200 μl of PBS with Vortex Oscillator to get the resuspended platelets.

3.5.2. Mark of tubulin

3.5.2.1. Mark of α-tubulin

(1) Resuspended platelets were taken into labeled flow tubes, and 20 μl of mouse anti-human CD61-phycoerythrin (PE) monoclonal antibody was added into the tubes respectively to mark the platelets. Samples were incubated for 20 min at 25°C in a dark place.

(2) The samples were washed with 2 ml of PBS and centrifuged at 1000 g for 10 min. Supernatant was discarded and the sediment was mixed well with 200 μl of IntraPrep Reagent 1: Fixation. Then they were incubated for 20 min at 25°C in a dark place.

(3) The samples were washed with 2 ml of PBS and centrifuged at 1000 g for 10 min again. Supernatant was discarded and the sediment was mixed well with 200 μl of IntraPrep Reagent 2: Permeabilization. Then they were incubated for 15 min at 25°C in a dark place.

(4) The samples were washed with 2 ml of PBS and centrifuged at 1000 g for 10 min. Supernatant was discarded and the sediment was mixed well with 100 μl of PBS to resuspend platelets.

(5) Then the samples were mixed with 2 μl of rabbit anti-alpha tubulin polyclonal antibody, and incubated for 15 min at 25°C in a dark place.

(6) The samples were washed with 1 ml of PBS and centrifuged at 1000 g for 10 min. Supernatant was discarded and the sediment was mixed well with 100 μl of PBS to resuspend platelets.

(7) Then the samples were mixed with 2 μl of goat anti-rabbit IgG-fluorescein isothiocyanate (FITC), and incubated for 20 min at 25°C in a dark place.

(8) The samples were washed with 1 ml of PBS and centrifuged at 1000 g for 10 min. Supernatant was discarded and the sediment was mixed well with 1 ml of PBS to resuspend platelets.

3.5.2.2. Mark of β-tubulin. Steps of mark of β-tubulin are similar to step 3.5.2.1. The exclusive difference: 2 μl of rabbit anti-alpha tubulin polyclonal antibody
in step of 3.5.2.1(5) was replaced with 2 μl of rabbit anti-beta tubulin polyclonal antibody in this step.

3.5.3. Detection the expression of α-tubulin and β-tubulin in platelets with flow cytometry

Detection of platelets antigens (IgG-FITC/CD61-PE) was established using the detection software (EXPO32 ADC). The detection scheme was made zero by detecting a control tube. The positive rate of the FITC channel was made zero by adjusting the voltage of the fast compensation channel. Finally, the detection protocol was saved. In addition, the sample was stopped loading after collecting 50,000 cells.

Firstly, samples in control tubes were replaced in the sample stage, and results were saved. Secondly, test tubes were placed in the stage respectively, then results and figures were saved. Results of flow cytometry were processed by EXPO32 ADC and the expression intensity of α-tubulin and β-tubulin was expressed by mean fluorescence intensity (MFI).

3.5.4. Statistical process

All statistical analysis in our work were performed using SPSS 18.0 software and all data were conformed to normal distribution by K–S test. Statistical results were expressed as mean ± standard deviation (x ± s). The mean was compared using one-way ANOVA for statistical analysis. If P < .05, the difference would be statistically significant.

4. Results

4.1. Basic information

By one-way ANOVA, it was showed that there were no significant differences in age and sex distribution among the group of healthy controls, the group of IDA patients with normal platelet counts and the group of IDA patients with increased platelet counts (P > .05). The RBC, HGB, HCT, MCV, MCH, MCHC, serum iron (Fe) and ferritin in two group of IDA patients were significantly less than those in the group of healthy controls. But there was no significant difference between two groups of IDA patients (P > .05) (Table 3).

4.2. Count of platelets

4.2.1. Counted by LH780 hematology analyzer

Count of healthy controls was (193.46 ± 46.67), and count of two group of IDA patients was (198.11 ± 62.41) (normal platelet counts) and (450.29 ± 57.08) (increased platelet counts). By one-way ANOVA, it was showed that platelet counts in peripheral blood of IDA patients with increased platelet counts was significantly higher than that of healthy controls and IDA patients with normal platelet counts (P < .01). Meanwhile, there was no significant difference in platelet counts between group of healthy controls and IDA patients with normal platelet counts (P > .05) (Figure 3).

4.2.2. Count with hemocytometer under a microscope

Count of healthy controls was (197.44 ± 60.38), and count of two group of IDA patients was (195.71 ± 66.83) (normal platelet counts) and (456.76 ± 44.13) (increased platelet counts). By one-way ANOVA, it was showed that platelet counts of IDA patients with increased platelet counts was significantly higher than that of healthy controls and IDA patients with normal platelet counts (P < .01). Meanwhile, there was no significant difference in platelet counts between group of healthy controls and IDA patients with normal platelet counts (P > .05) (Figure 4).

4.2.3. Comparison of platelet counts between LH-780 hematology analyzer and hemocytometer

Statistical analysis was performed by using one-way ANOVA. The result was showed that there were no significant differences in platelet counts of these three groups with LH-780 hematology analyzer and microscope (all P > .05) (Table 4 and Figure 5).

Table 3. Basic information of healthy controls, IDA patients with normal platelet counts and IDA patients with increased platelet counts.

| Items               | Healthy control | IDA patients with normal platelet counts | IDA patients with increased platelet counts |
|---------------------|-----------------|------------------------------------------|--------------------------------------------|
| Number of cases     | 45              | 34                                       | 45                                         |
| Gender (male/female)| 14/31           | 11/23                                    | 15/30                                      |
| Age (years old)     | 34.18 ± 9.55    | 35.18 ± 7.62                             | 32.55 ± 7.96                               |
| RBC (x10^12/L)      | 4.28 ± 0.44     | 3.34 ± 0.30                              | 3.51 ± 0.42                                |
| HGB (g/L)           | 129.44 ± 10.60  | 80.35 ± 15.78                            | 82.27 ± 13.42                              |
| HCT                 | 0.411 ± 0.027   | 0.275 ± 0.045                            | 0.278 ± 0.038                              |
| MCV (fL)            | 89.06 ± 6.04    | 69.44 ± 5.80                             | 70.91 ± 5.39                               |
| MCH (pg)            | 29.98 ± 1.78    | 22.70 ± 2.60                             | 22.10 ± 2.98                               |
| MCHC (g/L)          | 342.34 ± 11.61  | 295.79 ± 14.76                           | 296.90 ± 13.86                             |
| Fe (μmol/L)         | 18.50 ± 5.52    | 5.28 ± 2.71                              | 4.98 ± 2.29                                |
| Ferritin (ng/ml)    | 92.49 ± 47.46   | 9.48 ± 1.93                              | 8.79 ± 2.91                                |

Figure 3. Peripheral blood platelet counts by using LH-780 hematology analyzer.

*Comparison with healthy controls and IDA patients with normal platelet counts, P < .01.
4.3. Expressions of α-tubulin and β-tubulin within platelets

It was showed by flow cytometry that (Figures 6 and 7), the MFI of α-tubulin within platelets of these three groups were respectively (81.18 ± 20.97) (healthy controls), (80.56 ± 16.09) (IDA with normal platelet counts) and (62.95 ± 20.53) (IDA with increased platelet counts). Statistical analysis was performed by using one-way ANOVA. The result was showed that MFI of α-tubulin in group of IDA with increased platelet counts was significantly less than that in group of healthy controls and IDA with normal platelet counts ($P < .01$). In addition, there was no significant difference in MFI of α-tubulin between group of healthy controls and group of IDA with normal platelet counts ($P > .05$) (Tables 5–7).

As for MFI of β-tubulin, MFI in group of healthy controls was (27.76 ± 11.55), and MFI in group of IDA with normal platelet counts was (26.82 ± 8.56), and MFI of group of IDA with increased platelet counts was (17.55 ± 8.14). With one-way ANOVA, it was showed that MFI of β-tubulin in group of IDA with increased platelet counts was significantly less than that in group of healthy controls and IDA with normal platelet counts ($P < .01$). In addition, there was no significant difference in MFI of β-tubulin between group of healthy controls and group of IDA with normal platelet counts ($P > .05$) (Tables 5–7; Figure 8).

5. Discussion

The results of our work showed that a considerable number of IDA patients associated with increased platelet counts, which was consistent with the previous report [5]. Count of platelets in normal human is in a stable range and in a dynamic balance. Anyhow, the reason for the increase in platelet counts is nothing more than increased generation and/or the decreased destruction. As for the IDA patients associated with increased platelet counts, according to clinical experiences and literatures, we believed that it was majorly caused by increased platelets production. In addition, platelets production involved the structure and movement of cytoskeleton, and tubulin was an important part of the cytoskeleton [6].

Table 4. Comparison between platelet counts detected by using LH-780 hematology analyzer and hemocytometer under a microscope.

| Groups                        | LH-780 hematology analyzer | Hemocytometer under a microscope | $P$-value |
|-------------------------------|-----------------------------|----------------------------------|-----------|
| Healthy control               | 193.47 ± 55.15              | 197.44 ± 60.38                   | .745      |
| IDA patients with normal platelet counts | 198.11 ± 62.41              | 195.71 ± 66.83                   | .879      |
| IDA patients with increased platelet counts | 450.29 ± 57.08              | 456.76 ± 44.13                   | .549      |

Figure 4. Count of platelets with hemocytometer under a microscope.

*Comparison with group of healthy controls and IDA patients with normal platelet counts, $P < .01$.

Figure 5. Column diagram of comparison between platelet counts detected by using LH-780 hematology analyzer and hemocytometer under a microscope.
5.1. Molecular biology of tubulin

Tubulin is the main component of microtubules, and commonly there are three types of the protein, α-tubulin, β-tubulin and γ-tubulin [5]. The former two are the main tubulins belonging to spherical molecule. Furthermore, α-tubulin (450 amino acids) and β-tubulin (455 amino acids) have an almost identical three-dimensional structure that can be tightly bound to polymerize a dimer as a subunit for assembly of microtubules [7]. The homology of the amino acid sequences of α-subunit and β-subunit are about 40%. Both α-tubulin and β-tubulin have GTP binding sites. GTP combining with the site which is called as irreversible binding site on the α-subunit cannot be hydrolyzed and exchanged by GDP. By contrary, GTP and GDP can bind to the site of β-tubulin which has GTPase activity and the site on β-tubulin is called as exchangeable site [8, 19]. Besides, binding of GTP or GDP on the β-subunit will affect the stability of the microtubule dimer. The dimer that binds to GTP tends to be assembled into microtubules, however, the dimer that binds to GDP tends to separate microtubules [9]. Therefore, the cycle of GDP and GTP is essential to maintain the stability of microtubule.

Additionally, γ-tubulin is the other members of the tubulin family and plays an important role in nucleation and polar orientation of microtubules. It is mainly present in the microtubule nucleation area, such as cell centrioles and spindles [10]. It is found that there are other four kinds of tubulin (δ, ε, ζ and η) in eukaryotic cells, but compared with the former three kinds of tubulin, their structures and functions are more simple. It was showed that γ, δ, ε, ζ and η-tubulin were involved in the formation of nuclei. Since platelets were non-nuclei cells, the expression of γ, δ, ε, ζ and η-tubulin were not detected in our work.

5.2. The role of tubulin in formation and activation of platelets

Marginal bands composed entirely of microtubules beneath the plasma membrane are the requirement to
maintain discoid shape of resting platelets [11]. Through megakaryocyte microtubule system marked by the EB3 (end-binding protein 3)-GFP (green fluorescent protein) antibody, S. Patel-Hett observed the megakaryocyte microtubule movement, and examined its behavior in living platelets releasing from megakaryocytes. The EB3-GFP antibody in resting platelets over time revealed about multiple sites and two-way active patterns on the microtubule coil, providing a clear indication for microtubule assembly in the resting platelets microtubule coil. It is found that marginal band in the resting platelets contains multiple highly dynamic microtubules with mixed polarity, including unipolar and bipolar dynamic structures. In the unipolar dynamic structure, the polymerized microtubules only moved in one direction on the marginal band, while in the bipolar dynamic structures, tubulin was polymerized and separated in a clockwise and counterclockwise direction respectively around the marginal band. Dynamic microtubule balance produces different platelets function and alters the physiological processes of its cytoskeleton, such as activation and adhesion of platelets, and formation of filopodia, etc. Enhanced enhancement of EB3-GFP activity showed a significant increase in polymerized microtubules, and partial platelets even outward to form filopodia. Thus, dynamic balanced

Table 5. Expression of tubulin in platelets within IDA patients with increased platelet counts and healthy controls.

| Tubulin   | IDA patients with increased platelet counts | Healthy controls | P-value |
|-----------|--------------------------------------------|------------------|---------|
| Cases     | 45                                         | 45               | –       |
| α-Tubulin | 62.95 ± 20.53                              | 81.18 ± 20.97    | .000    |
| β-Tubulin | 17.55 ± 8.14                               | 27.76 ± 11.55    | .000    |

Table 6. Expression of tubulin in platelets of IDA patients with increased platelet counts and IDA patients with normal platelet counts.

| Tubulin   | IDA patients with increased platelet counts | IDA patients with normal platelet counts | P-value |
|-----------|--------------------------------------------|----------------------------------------|---------|
| Cases     | 45                                         | 34                                     | –       |
| α-Tubulin | 62.95 ± 20.53                              | 80.56 ± 16.09                          | .000    |
| β-Tubulin | 17.55 ± 8.14                               | 26.82 ± 8.56                           | .000    |
marginal band may be involved in the activation of resting platelets [11].

Through diameter of microtubule coil within resting platelets, it is suggested that the shrinkage of the microtubule coil indicates the caducity of platelets. The tubulin is involved in triggering activation of platelets structural changes, meanwhile, dynamic balance of microtubule was significantly correlated with platelets maturation, which appeared as more mature platelets with smaller diameters of coli. The coil diameter of the newly platelets is about 15% larger than that of the platelets coil of the control group [11]. Therefore, it is thought that the newly produced platelets have the reshaping cytoskeleton of microtubule. The polymerization of tubulin within the marginal band was matched with the degree of platelets maturation.

### 5.3. Relationship between tubulin and IDA

It was found that in the concentration of Fe$^{3+}$ could interfere with the process of polymerizing tubulin and its related proteins in rat brain to form microtubules. The microtubule structure was normally polymerized at Fe$^{3+}$ concentration of 10 µM (micromoles per liter). At Fe$^{3+}$ concentration of 100 µM, tubulin assembly and microtubule polymerization were abnormal. At 250 µM Fe$^{3+}$ concentration, the microtubules lost the normal structure, and the coil was completely entangled together, and lost the tubular structure. Even more, at 500 µM Fe$^{3+}$ concentration, it occurred rare protein abnormalities and short microtubule-like structures. This study was to grope for the possible causes of structural abnormalities of microtubule in the brain in Alzheimer’s disease, by exploring the changes of the structure of microtubules and tubulin in the brain of rats with different concentrations of Zn$^{2+}$, Al$^{3+}$ and Fe$^{3+}$[12]. That was to say that it was not a study on changes of microtubule structure and tubulin with low concentration of Fe$^{3+}$ or iron deficiency. However, according to the process of tubulin assembly and the changes with different concentrations of Fe$^{3+}$, we hypothesized that Fe$^{3+}$ has different effects on the assembly of microtubules at different concentrations, and that different Fe$^{3+}$ concentrations may affect the activity of enzymes involved in microtubule assembly (e.g. GTPase, iron-ase, etc.).

### 5.4. Possible mechanism of IDA accompanied by thrombocytosis

In our work, the expression of tubulin within platelets of IDA patients with thrombocytosis was studied by measuring the fluorescence intensity of tubulin α and β. The experimental data showed that the expression of α-tubulin and β-tubulin within platelets of IDA with thrombocytopenia was significantly less than that in normal individuals and IDA patients with normal platelet counts. Platelets activation was closely related with microtubule movement and dynamic balance of polymerization and depolymerization of tubulin [8], as an example, downregulation of β-tubulin may inhibit thrombin-induced human platelets activation. From this, we hypothesized that platelets function was affected when lacking tubulin, such as the activation and adhesion of platelets, the formation of

| Tubulin  | Healthy controls | IDA patients with normal platelet counts | P-value |
|---------|-----------------|-------------------------------------------|---------|
| Cases   | 45              | 34                                        |         |
| α-Tubulin | 81.18 ± 20.97  | 80.56 ± 16.09                             | .886    |
| β-Tubulin | 27.76 ± 11.55  | 26.82 ± 8.56                              | .691    |

Figure 8. Column diagram of comparison between MFI of alpha tubulin and beta tubulin within platelets.

*Comparison with group of healthy controls and IDA patients with normal platelet counts, $P < .01$. 

Table 7. Expression of tubulin in platelets of IDA patients with normal platelet counts and healthy controls.
counts, of which with IDA were not associated with increased platelet counts in IDA patients. This phenomenon suggests that downregulation of tubulin is likely to be involved in the process of increasing platelet counts in IDA patients.

The results of this study showed that some patients with IDA were not associated with increased platelet counts, of which α-tubulin and β-tubulin expression was significantly higher than that associated with IDA patients with thrombocytosis, but no statistical difference with healthy controls. We hypothesized that the structure of α-tubulin and β-tubulin may be different between IDA patients with normal platelet counts and patients with thrombocytosis. Thus, in the case of the same iron deficiency, the effect of iron deficiency on tubulins with different structures may be different, which needs further study.

6. Conclusion

The expression of α-tubulin and β-tubulin within platelets in IDA patients with thrombocytosis is significantly less than that in patients with normal platelet counts and healthy controls, and the platelet counts in peripheral blood of patients with iron deficiency anemia were increased. Meanwhile, downregulation of tubulin within platelets, which may be one of mechanisms of dysfunction of platelets, resulting compensatory thrombocytosis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Appendix

Informed Consent

Research background

Your whole blood will be used by professor Yin of the Second Affiliated Hospital of Shantou University Medical College.

This study was approved by the ethical review committee of the Second Affiliated Hospital of Shantou University Medical College. This form gives you important information about the study. It describes the purpose, process and method of the study, and please take time to consider this information carefully. If you choose to take part in the study, you will be asked to sign this form.

Research purposes

In order to inquire into the pathogenesis of increased platelet counts in peripheral blood of patients with iron deficiency anemia (IDA), the phenomenon of thrombocytosis was confirmed, and then the expression of tubulin within platelets from IDA patients was investigated.

Research process and methods

Your whole blood sample (about 5 mL) will be drawn and the number of platelets will be counted then tubulin within platelets will be detected.

Research significance

We will explore the mechanisms of why some IDA patients associate thrombocytosis.

Privacy policy

Researchers will safeguard your privacy.

Statement

Taking part in this study is completely voluntary. You do not have to participate if you don’t want to. You can refuse us without any reasons.

Signature

I understand the information printed on this form. My questions have until now been answered. I agree to take part in this study.

Signature