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• *BASIC RESEARCH* •

Effect of Haimiding on the functioning of red cell membrane of FC and H_{22} tumor-bearing mice

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Abstract

AIM: To study the effect of Haimiding on the functioning of red cell membrane of FC and H_{22} tumor-bearing mice.

METHODS: The membrane fluidity of red cells is measured with DPH fluorescence probe as a marker; the amount of red cell membrane proteins is measured using polyacrylamide gel electrophoresis; the amount of sialic acid (SA) on the surface of red cell membrane and the sealability of these cells are measured using colorimetric analysis.

RESULTS: Haimiding can lower the membrane fluidity of red cells in tumor-bearing mice and the amount of their membrane proteins, while increasing the amount of sialic acid in the membrane of red cells in these mice and enhancing the ability of the membrane of their red cells to reseal.

CONCLUSION: The anti-tumor effect of Haimiding on tumor-bearing mice is due to its ability to improve and restore the functions of the membrane of their red cell and to enhance the immune effect of the organisms.

Key words: Membrane function; FC; H_{22}

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INTRODUCTION

Haimiding is a compound anticancer preparation combining Chinese and Western medicines. It is prepared from the anticancer drug 5-Fu (5-fluorouracil) plus extracts from Chinese medicines including *Sargassum fusiforme*, *Ecklonia kurome*, *Astragalus chrysopterus*, and *Sophora flavescens*. Findings from our earlier studies show that Haimiding has some killing power on 8 kinds of human cancerous cells, namely, human oral epithelial KB, human esophageal carcinoma E_{ca}-109, human proventriculus BGC-823, human pulmonary adenocarcinoma A_{549}, human colon HCT-8, human breast MCF-7, human ovary A_{2780}, and human liver Bel-7402. Its inhibitory effect is most prominent on BGC-823, E_{CA}-109, and HCT-8 cells. It clearly can inhibit the formation of BGC-823, E_{CA}-109, and HCT-8 colonies with the effect on E_{CA}-109 colonies being the strongest. It has a clear inhibitory effect on the growth of FC tumors in mice, and can significantly prolong the life span of H_{22} tumor-bearing mice[1]. The cell membrane, which constitutes a barrier between the cytoplasm and the tissue, contains many kinds of proteins[2-5] and receptors[6-8], which are related to the functioning of the organism and are closely related to the genesis and development of tumors[10-14]. Based on these, we tried in the present study to observe the effect of Haimiding on the functioning of red cell membrane of tumor-bearing mice, aiming to explicate the mechanism of the anticancer effect of Haimiding.

MATERIALS AND METHODS

Materials

Test animals White mouse (of the Kunming strain), acquired from Laboratory Animal Center of Heilongjiang University of Chinese Medicine (certificate no.: Heilongjiang animal No. 00101003), half-male and half-female, weighing 20±2 g.
Tumor strains FC and H_{22} tumor-bearing mice, acquired from the Institute of Tumor Research, Harbin Tumor Hospital.
Major reagents Haimiding is provided by the Institute of Materia Medica, Harbin Commercial University (lot no.: 20010517); 5-FU, acquired from Shanghai No. 12 Pharmaceutical Company (lot no.: 20010601); DPH (1,6-diphenyl-1, 3, 5-hexatriene), product of Fluke AG.
Main equipment RF-540 fluorescence spectrophotometer, produced in Shimazu, Japan; thermostatic water bath, acquired from Xiamen Medical Electronic Instruments.

Methods

Development of the mouse tumor model (1) Drawing abdominal dropsy. The mouse was killed by taking apart at the joint of its cervical vertebra 7 d after tumor cells were transplanted. The animal was then fixed on a wax plate. After sterilization, the abdominal skin was cut open and peeled off. A syringe sterilized under high temperature was

used to draw the abdominal dropsy, which was put in a germ-free container surrounded by ice cubes for storage. In addition, a small amount of abdominal dropsy was drawn and put in a test tube to be used for observing and counting the cells. A drop of the abdominal dropsy remaining in the syringe was put on a slide, which was smeared and dyed with Wright's staining, and the cells were assorted and counted. The sample was to be used only when 97% or more of the cells were cancerous. (2) Inoculation. The abdominal dropsy was diluted with normal saline to 1∶4. The FC tumor-bearing mouse was created by injecting the dropsy at the animal's armpit, and the H_{22} tumor-bearing mouse was created by injecting the dropsy at the animal's abdomen.

Grouping and administration of drugs (1) Grouping. The mice were randomly divided into 6 groups, with a normal group, a negative control group, a positive control group, and another 3 groups to be given high, medium, and low dosages of Haimiding. Each group consists of 10 mice. (2) Administration of drugs. Twenty-four h after tumor cells were transplanted into the mice, drugs were administered by abdominal injection once a d for 7 consecutive d. The positive control group was given 25 mg/kg of 5-FU, the treated groups were given 25, 50, and 100 mg/kg of Haimiding, and the negative control group was given normal saline of the same volume. On the day following the last day of drug administration, the animals were killed, and relevant measurements were taken.

Effect of Haimiding on the immunological adhesiveness of red in tumor-bearing mice to tumor cells After the animal was killed, blood was drawn, centrifuged, and rinsed to make a red cell suspension (1×10^8 piece/mL) to be used later. When the experiment was conducted, H_{22} tumor cells were drawn from a 7-d old abdominal cavity of the mouse and rinsed twice with Hanks' solution to make a tumor cell suspension (1×10^6/mL). 0.1mL of this suspension was taken and an equal volume of fresh guinea pig (or human) blood was added to the sample. The sample was then set in water bath at 37 ℃ for 1 h, centrifuged and rinsed twice, with the supernatant being discarded to yield serum-sensitized cancer cells. 0.05 mL of the red cell suspension was set in water bath at 37 ℃ for 30 min, and 0.25% glutaraldehyde was added to fix the solution, a slide was smeared with the preparation and dyed using Wright's staining. At this point, the tumor cells appeared to be blue, while red cells were red. One tumor cell joining the 3 or more red cells made up a tumor-red cell wreath. The percentage of tumor-red cell wreath was calculated.

Effect of Haimiding on the fluidity of red cell membrane of tumor-bearing mice A 2 mL 0.1% heparin was added to each of the several 10-mL test tubes. Three drops of blood was drawn from the experiment animal by cutting its tail. The blood were put in those tubes, mixed, and centrifuged for 10 min at 3 000 r/min. After the supernatant was discarded, the residue was rinsed 3 times with isotonic phosphoric acid buffer solution, and the red cells were counted. With the number of red cells thus obtained, a red cell suspension with 4×10^7 piece/mL was made. A 2 mL of the 4 mL red cell suspension was taken and added to a test tube as the blank tube, while the test tube containing the remaining 2 mL of the red cell suspension was used for reference. A 2 mL of DPH probe solution was added to the reference tube, while the same amount of isotonic PBS buffer solution was added to the blank tube. Solution in the two tubes was mixed and incubated for 30 min under a temperature of 25 ℃ and then centrifuged for 10 min at 3 000 r/min. The remaining DPH probe solution was discarded, and the residue was rinsed twice with isotonic PBS buffer solution and then diluted into 4 mL of cell suspension with isotonic PBS buffer solution. Immediately after this, the fluorescence polarization of the residue was measured. RF-540 fluorescence spectrophotometer with xenon lamp as light source was used to measure the intensity P of fluorescence polarized light both when it was parallel to and when it was perpendicular to the direction of vibration of the excitation polarized light, at a fluorescence excitation wavelength of 362 nm, a radiation wavelength of 432 nm, and under a temperature of 25 ℃. Then the micro viscosity was calculated.

$$\eta = \frac{2P}{0.46-P}$$

Effect of Haimiding on the membrane protein content in the red cells of tumor-bearing mice: Preparation of red cell membrane

Whole blood

↓ centrifuged at 3 000 r/min for 10 min, rinsed twice with 1∶3 saline, and suspended in 1∶1 saline

Red cell suspension

↓ 5p 8.4 phosphoric buffer solution (1∶30) was added at 4 ℃ and hemolysis was allowed to proceed for 1 h.

Hemolyzed solution of red cells

↓ centrifuged at 2 000 r/min at 4 ℃ for 40 min; the supernatant was discarded; the residue was rinsed 3 times and then suspended in 5p8.4 phosphoric acid buffer solution.

Membrane suspension

The concentration of red cell membrane protein was measured according to Lowry's rule. DSD-polyacrylamide gel electrophoresis was performed to separate the cell membrane protein and measure its content.

Gel, gel plate, gel repository solution, electrolytic tank buffer solution, and dyeing solutions I and II were prepared as described in literature[15]. SDS was used to solubilize membrane so as to separate the proteins from the membrane. The sample was loaded in the following manner: 50 μL of membrane sample was sucked with a microsyringe. After the sample was loaded, it was immediately covered with electrolytic tank buffer solution, and tap water and cooling water tube of the electrophoresis tank were turned on. Electrophoresis was conducted for 3.5 h with the upper tank as the cathode and the lower one as the anode, the voltage adjusted to 50 V and electric current to 30 mA. After electrophoresis, the gel was stripped off and the sample was dyed. The gel plate was placed on a CS-930 double-wavelength scanner and scanned at 560 nm to yield an electrophoretogram of the membrane protein, and then the percentages of the components of the membrane protein were calculated.

Effect of Haimiding on the sialic acid content on the

Table 1 Steps for measuring the activity of the sialic acid content on the surface of the red cell membrane

Reagent	Blank	1	2	3	4	5	6	7
N-acetyl neuraminic acid Standard solution (mL)	0	0.05	0.1	0.2	0.3	0.4	0.5	0.8
N-acetyl neuraminic acid (μg)	0	5	10	20	30	40	50	80
Distilled water (mL)	1	0.95	0.9	0.8	0.7	0.6	0.5	0.2
Bialsche reagent (mL)	1	1	1	1	1	1	1	1
Boil for 12 min in water bath, then cool for 3 min with ice water								
Pentanol (mL)	5	5	5	5	5	5	5	5
Shaken thoroughly and then centrifuged for 10 min at 1 000 r/min; took the pentanol phase for colorimetric analysis at a wavelength 569 nm								

Table 2 Steps for measuring the activity of the oxidoreductase NADH-cell pigment C

Reagent (mL)	No saponin added (-S)		Saponin added (+S)	
	Blank	Sample	Blank	Sample
0.6 mg protein/mL Membrane suspension	0.6	0.6	0.6	0.6
PBS (5p8.4)	2.4	2.2	2.2	2.0
0.1% saponin	/	/	0.2	0.2
5 mmol/L $K_3Fe(CN)_6$	/	0.1	/	0.1
Let the solution stand for 10 min, added NADH, and recorded the result after 1 min 6 mmol/L NADH	/	0.1	/	0.1

surface of the red cell membrane of tumor-bearing mice Red cell membrane was prepared as before.

The standard curve was plotted.

0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.8 mL of *N*-acetyl neuraminic acid was added to 8 test tubes each, which was to say the test tubes contained 0, 5, 10, 20, 30, 40, 50 and 80 μg respectively of *N*-acetyl neuraminic acid. Then the optic density (*A*) of each tube was measured according to the procedures in the following table, and a standard curve was plotted for the *N*-acetyl neuraminic acid content (Table 1).

Measurement of sialic acid content in red cell membrane Bialsche reagent was added to 1 mL of the membrane suspension prepared earlier (0.6 mg protein per millimeter membrane liquid) to measure directly the sialic acid (SA) content (by measuring the absorbance, or *A*). The procedure was the same as above.

Effect of Haimiding on the sealability of the red cell membrane of tumor-bearing mice

Preparation of closed membrane (isotonic closed membrane) from red cells First, an unclosed membrane preparation was made in the same manner as before. The membrane was then rinsed once with isotonic phosphoric acid buffer solution, centrifuged for 40 min at 2 000 r/min, and then placed in isotonic solution, which was kept at 37 ℃ for 1 h to yield closed membrane. The activity of the oxidoreductase NADH-cell pigment C was measured by the method described in literature[16]: a 0.6 mL sample of membrane (membrane fluid) was put in a colorimetric cup, and reactants were added as shown in the Table 2. After the reactants were each mixed with the sample, their colors were compared at a wavelength of 420 nm. The time at which NADH was added was taken as time zero, and *A* was recorded once every min for 6 min after the reaction starts. The reaction was to take place under room temperature (25 ℃). Colorimetric analysis was performed. The procedure was the same as above except that NADH was not added. Specific steps were shown in Table 2.

Calculation of sealability The sealability of membrane (%) was calculated according to the formula below:

$$\text{Sealability (\%)} = \frac{\text{Activity when surfactant is added - Activity when no surfactant is added}}{\text{Activity when surfactant is added}} \times 100\%$$

Statistical analysis

t test was performed on data obtained from the experiments, with the results expressed in the format mean±SD.

RESULTS

Effect of Haimiding on the immunological adhesiveness of red cells in tumor-bearing mice to tumor cells

Experimental results showed that the immunological adhesiveness of red cells in tumor-bearing mice, as measured by the rate of wreaths, was weaker than that in the control group, with the difference being more prominent with H_{22} mice than with FC mice. And the rate of wreaths was significantly higher ($P<0.01$) in the treated group than in the group that was administered normal saline, with the level for FC group that was treated being close to the normal, while that for the 5-Fu group showing no clear increase, or even showing some trend toward decreasing (Tables 3, 4).

Table 3 Effect of Haimiding on the immunological adhesiveness of red cells of FC mice to tumor cells (mean±SD)

Group	Number of animals (*n*)	Route of administration	Dosage (mg/kg)	Rate of wreaths (%)
Control	10	Ip		36.36±5.42
Saline	10	Ip	Equal volume	10.46±3.55
5-Fu	10	Ip	25	8.41±2.56
Haimiding	10	Ip	50	28.96±4.41[b]

[b]$P<0.01$ *vs* the saline group.

Table 4 Effect of Haimiding on the immunological adhesiveness of red cells of H_{22} mice to tumor cells mean±SD

Group	Dosage (mg/kg)	Route of administration	No. of animals (*n*)	Rate of wreaths (%)
Control		Ip	10	36.36±5.42
Saline	Equal volume	Ip	10	5.10±2.28
5-Fu	25	Ip	10	5.96±3.15
Haimiding	50	Ip	10	25.68±4.13 [b]

[b]$P<0.01$ *vs* the saline group.

Effect of Haimiding on the fluidity of intact red cell membrane of tumor-bearing mice

From Tables 5, 6 it can be seen that, compared to the control, the micro viscosity of red cells in tumor-bearing mice had increased, and the membrane fluidity had decreased. But after treatment, the microviscosity of red cells in the two strains of tumor-bearing mice decreased to different degrees, which means membrane fluidity had increased. The effect was the most prominent with the group treated with medium dosage of Haimiding ($P<0.01$).

Effect of Haimiding on the membrane protein content in the red cells of tumor-bearing mice

From Tables 7, 8 it can be seen that the percentage of polymers formed on the red cell membrane of the tumor-bearing mice of the saline group was higher than that in the control group, and that the level of polymers decreased significantly after the animals had been treated with medium dosage of Haimiding ($P<0.01$). For different tumor-bearing organisms, the effect of high dosage of Haimiding differed to some extent. The results also showed that for the same dosage, the effect for H_{22} mice was more prominent than that for FC mice.

Table 7 Effect of Haimiding on the membrane protein content in the red cells of FC mice

Group	Number of animals (*n*)	Route of administration	Dosage (mg/kg)	Membrane protein content %
Control	10	Ip	Equal volume	0.281±0.051
Saline	10	Ip	25	0.332±0.094
5-Fu	10	Ip	100	0.278±0.041
Haimiding	10	Ip	50	0.241±0.037[a]
Haimiding	10	Ip	25	0.187±0.042[b]
Haimiding	10	Ip		0.296±0.040

[a]$P<0.05$, [b]$P<0.01$ *vs* the saline group.

Table 8 Effect of Haimiding on the membrane protein content in the red cells of H_{22} mice

Group	Number of animals (*n*)	Route of administration	Dosage (mg/kg)	Membrane protein content %
Control	10		Equal volume	0.281±0.051
Saline	10	Ip	25	0.298±0.088
5-Fu	10	Ip	100	0.219±0.089
Haimiding	10	Ip	50	0.237±0.050
Haimiding	10	Ip	25	0.143±0.064[b]
Haimiding	10	Ip		0.189±0.094[a]

[a]$P<0.05$, [b]$P<0.01$ *vs* the saline group.

Effect of Haimiding on the sialic acid content on the surface of the red cell membrane of tumor-bearing mice

The results are shown in Tables 9, 10. From the study on the effect of Haimiding on the SA content in red cell membrane of tumor-bearing mice, it was found that the SA content for tumor-bearing mice especially for those of the H_{22} strain was significantly lower than that for normal mice. After Haimiding was administered, the SA content in the red cell membrane of tumor-bearing mice increased to different degrees, with the medium- and high-dosage groups markedly different from the saline group

Table 5 Effect of Haimiding on the fluidity of intact red cell membrane of FC mice

Group	Number of animals (*n*)	Route of administration	Dosage (mg/kg)	Fluorescence polarization (*P*)	Micro viscosity, η (*P*)	Membrane lipid fluidity (LFU)
Control	10		Equal volume	0.2006±0.0728	1.7557±0.9616	7.4472±2.3273
Saline	10	Ip	25	0.2438±0.0122	1.9458±0.2367	4.3134±1.4378
5-Fu	10	Ip	100	0.2169±0.0269	1.8183±0.3799	6.0234±1.9430[a]
Haimiding	10	Ip	50	0.215±0.0242 [b]	1.796±0.1414 [a]	6.1913±1.9348 [a]
Haimiding	10	Ip	25	0.210±0.0137 [b]	1.701±0.2063 [a]	6.4966±1.9687 [b]
Haimiding	10	Ip		0.2203±0.0338	1.7330±0.4404	5.7670±1.6962 [a]

[a]$P<0.05$, [b]$P<0.01$ *vs* the saline group.

Table 6 Effect of Haimiding on the fluidity of intact red cell membrane of H_{22} mice

Group	Number of animals (*n*)	Route of administration	Dosage (mg/kg)	Fluorescence polarization (*P*)	Micro viscosity, η (*P*)	Membrane lipid fluidity (LFU)
Control	10			0.2006±0.0728	1.7557±0.9616	7.4478±2.3273
Saline	10	Ip	Equal volume	0.2430±0.03017	2.1378±0.6186	4.3559±1.4051
5£-Fu	10	Ip	25	0.2275±0.0182	1.9760±0.6738	5.2606±1.5472
Haimiding	10	Ip	100	0.2331±0.0211	2.0819±0.1772	4.9153±1.5360
Haimiding	10	Ip	50	0.1770±0.0567[b]	1.2775±0.4164[b]	11.0370±3.4489[b]
Haimiding	10	Ip	25	0.2111±0.0223[a]	1.8176±0.2106	6.4776±2.0895[a]

[a]$P<0.05$, [b]$P<0.01$ *vs* the saline group.

(P<0.01), and the effect on the medium-dosage group being the greatest.

Table 9 Effect of Haimiding on the sialic acid content on the surface of the red cell membrane of FC mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	A
Control	10	Ip		0.183±0.068
Saline	10	Ip	Equal volume	0.087±0.026
5-Fu	10	Ip	25	0.065±0.030
Haimiding	10	Ip	100	0.139±0.049 [b]
Haimiding	10	Ip	50	0.148±0.032 [b]
Haimiding	10	Ip	25	0.095±0.028

[b]P<0.01 *vs* the saline group.

Table 10 Effect of Haimiding on the sialic acid content on the surface of the red cell membrane of H_{22} mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	A
Control	10	Ip		0.183±0.068
Saline	10	Ip	Equal volume	0.062±0.027
5-Fu	10	Ip	25	0.064±0.034
Haimiding	10	Ip	100	0.119±0.040 [b]
Haimiding	10	Ip	50	0.125±0.044 [b]
Haimiding	10	Ip	25	0.081±0.023

[b]P<0.01 *vs* the saline group.

Effect of Haimiding on the sealability of the red cell membrane of tumor-bearing mice

The results in Tables 11, 12 show that the sealability of the red cell membrane of tumor-bearing mice was lower than that for normal mice. The level of sealability remains constant even when positive control drug was administered, but the administration of Haimiding could raise the capacity of red cells to reseal. The improvement was significant (P<0.01) for all 3 groups administered with different dosages.

Table 11 Effect of Haimiding on the sealability of the red cell membrane of FC mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	Sealability (%)
Control	10	Ip		70.6±4.05
Saline	10	Ip	Equal volume	54.6±2.21
5-Fu	10	Ip	25	52.0±3.39
Haimiding	10	Ip	100	63.5±3.11[b]
Haimiding	10	Ip	50	65.9±2.60[b]
Haimiding	10	Ip	25	58.4±2.05[b]

[b]P<0.01 *vs* the saline group.

Table 12 Effect of Haimiding on the sealability of the red cell membrane of H_{22} mice

Group	Number of animals (n)	Route of administration	Dosage (mg/kg)	Sealability (%)
Control	10	Ip		70.2±2.23
Saline	10	Ip	Equal volume	53.7±2.30
5-Fu	10	Ip	25	54.2±3.27
Haimiding	10	Ip	100	62.2±2.71[b]
Haimiding	10	Ip	50	61.4±3.44[b]
Haimiding	10	Ip	25	57.6±2.54[b]

[b]P<0.01 *vs* the saline group.

DISCUSSION

The cell membrane is an important structure indispensable for all cells. It has extremely important biological functions. The immunity of the cell and tumor genesis have all to do with the cell membrane. The immune effect of red cells is realized through the CR1 receptors on the membrane of these cells[18,19]. The new concept "Red-Cell Immune System" was first proposed in 1981 by the American scholar Siegel. Through extensive research in the last two decades, scholars in China and abroad have discovered that red cells, which have their origin in hemopoietic stem cells, have the most complete immunizing functions in the human body. They contain many substances that have to do with immunity and are capable of self-adjustment and self-control as well as play a role in the organism's immunological adjustment and control. Red cells are capable of recognizing, adhering to, and killing antigens, transmitting information about the antigens, and eliminating the immunity-inhibiting substances in circulation. Clinically, immunological defects of red cells play an important role in the pathogenesis of many diseases especially in the genesis of tumors.

In experiments we did in the past, we discovered that Haimiding can significantly improve the immunological functions of the red cells in tumor-bearing mice by significantly increasing the wreath rate of C3b receptors on red cells and the activity of promoters of immunological adhesiveness while significantly decreasing the wreath rate of immunological compounds in red cells and the activity of the inhibitors of immunological adhesiveness. In all 4 of these indices, Haimiding demonstrates an immune effect, which is not possessed by the chemotherapeutic drug 5-Fu. According to research results from recent years, red cells can immunologically adhere to various kinds of tumor cells, and under electron microscope it can be observed that tumor cells are damaged at points where they make contact with red cells, suggesting that red cells play the role of effector cells in the immune reactions against tumors. If there are autoantibodies for red cells in the serum of patients afflicted with tumors, this function of red cells is weakened[19,20]. In our experiments we also discovered that the capacity of red cells to adhere to H_{22} tumor cells decreases significantly in tumor-bearing mice as compared to normal mice, but this index is significantly improved for the groups which are administered Haimiding, while the administration of 5-Fu does not yield this kind of effect.

Patients afflicted with tumor often are also anemic[21-23]. At the same time, both the number and the activity of the CR1 on their red cells decrease significantly, resulting in low immune efficacy of their red cells. The immune efficacy of red cells in metastatic cancer patients is even lower, but after operation on the digestive tract, the immunological adhesiveness of these cells increases significantly. Research in recent years shows that red cells can immunologically adhere to tumor cells and prevent cancer cells from undergoing deuametastasis, which is the main route through which tumors spread. The number of red cells is 1 000 times that of white cells, and the chances for their getting into contact with tumor cells are much greater than that for white cells, so red cells play a more important role in preventing malignant tumor from metastasizing. Moreover, by

immunologically adhering to the CD2 on the membrane of T lymphocytes with the CD58 and CD59 on their own membrane, red cells can activate the immunological functions of T cells, which are expressed as an increase in the activity of IL-2R and in the secretion of γ-interferon. Red cells can release NK cell activators, thus increasing the activity of NK cells in killing cancer cells. Red cell membrane also contains receptors for chemotactic factors that can attract drifting chemotactic factors, thus reducing the concentration of chemotactic factors in blood circulation while enhancing the inflammation reaction of the tumorous tissue in question[24]. To sum up, red cells do not only perform antitumor immunological functions on their own, but play an important role in the overall control by the immune system of the human body that involves many kinds of cells and tissues and works through many routes, forming an antitumor immune control system.

Haimiding can enhance the capacity of red cells in tumor-bearing mice to adhere immunologically to tumor cells, which shows that it has an important effect on the spreading and metastasis of tumor. Furthermore, Haimiding can activate the immune system of the whole organism by enhancing the immunological effect of red cells, thus bringing about some overall therapeutic effects. The mechanism by which Haimiding enhances the immunological function of red cells may be as follows: The membranes of red cells contain some glycoprotein receptors, while *Astragalus chrysopterus*, a component of Haimiding, contains some glycoproteins. These glycoproteins can adhere to the glycoprotein receptors on the membrane of red cells, activating the CR1 on the membrane, thus enhancing the immunological effect of red cells.

The major types of membrane lipids of red cells include phospholipids (ovolecithin, cephalin, serine phosphatide, and sphingomyelin), cholesterols, and glycolipids. These lipids are arranged in a bi-layer molecular array, with the outer layer rich in ovolecithin. Phospholipids, cholesterols, and glycolipids all contain asymmetric hydrophilic and hydrophobic groups. In the bi-layer structure, the hydrophilic parts point outward, while the hydrophobic parts point toward the center of the structure. The two layers of lipids are fluid. Fluidity is an important mechanical property of cell membrane. All the normal physiological functions of red cells depend on the integrity of their membranes and the fluidity of the bi-layer structure of membrane lipids. The fluidity of red cell membrane has implications for the permeability, deformability, fragility, translocation of solutes and enzyme activity. It is directly related to the constituents of the serum especially those of serum lipoproteins[25]. Therefore, the study of the effect of Haimiding on the fluidity of red cell membrane can help us explore at the cellular and molecular levels the mechanism by which Haimiding works. As is well known, part of the molecular basis of malignant proliferation of cells is abnormality in the transmembrane signal transmission chain. The malignant transformation and redifferentiation of tumor cells may be related to the damage and repair respectively of the transmembrane signal transmission chain. Modifying or multiplying certain molecules involved may induce abnormalities in the chain and disorganization of the molecular processes, resulting in the loss of control of cell transformation and growth, which would lead to malignant tumors. Repairing or changing certain molecules involved can help restore the damaged transmembrane signal transmission chain, causing malignant tumor cells to redifferentiate and thus helping cancer cells to reverse their course of development. This procedure involves many kinds of molecules on the cell membrane, especially membrane proteins and membrane lipids. The canceration of cells and its reversion are both related to the fluidity of membrane[4,26]. Experiments in the present study show that Haimiding can lower the microviscosity of red cell membrane in tumor-bearing mice, resulting in the increase of membrane fluidity, and is thus helpful for adjusting the immunological functions of red cells in the organism and for restoring to normal the damaged transmembrane signal transmission chain, thus increasing the killing power of red cells on tumor cells.

After red cells are hemolyzed hypotonically and centrifuged at high speed, red cell membrane can be obtained. This is the so-called "ghost". Following treatment with detergent, SDS-polyacrylamide gel electrophoresis is performed on the sample. Red cell membranes are separated into different bands, which are treated with antibodies for shrinking protein, anchor protein, band 3 protein and band 4.1 proteins in immunoblot experiment. The results show that the polymers consist mainly of shrinking protein, anchor protein, and band 3 protein[14]. Thus far, we have discovered that the immune effect of red cells is mainly due to the protein molecules, such as CR_1 and CR_3, on their membranes. These proteins on the membranes of red cells are specially structured. They are not fixed on the membrane with a transmembrane structure of more than 20 hydrophobic amino acids, but are anchored on the membrane by covalently binding with some glycosyl phosphoric acid myoinositol, which increases their movability on the membrane, enabling these protein molecules, which are of a limited number, to be in direct contact with a large number of ligands to bring out their biological functions. However, in certain circumstances, such as when the organism is experiencing abnormality in its physiological functions, or when the cell membrane is damaged, or when shrinking proteins (i.e., band 1 and band 2 proteins) and band 4.1 proteins are cross-linked into high polymers, the composition of membrane proteins would change greatly. The cross-linking of membrane proteins directly affects the degree of freedom with which their spatial configurations can be changed, leading to changes in the mechanical properties of the membrane[27]. But findings from experiments for the present study show that Haimiding can improve the spatial stability of membrane proteins that have already undergone changes. From comparison with the saline group, it can be shown that Haimiding can significantly lower the production of high polymers of red cell membrane proteins of tumor-bearing mice, with the effect being significantly better with the groups administered medium dosage of Haimiding (50 mg/kg) than with the other two groups ($P<0.01$). Decrease in the amount of high polymers of membrane proteins can increase the fluidity of the membrane, decrease its microviscosity, accelerate the speed of blood flow, and increase the capacity of red cells to adhere immunologically to tumor cells. These findings

show that Haimiding can enhance the immunological effect of red cells of the organism by lowering the production of high, protein polymers on the membrane of these cells, thus enabling them to adhere immunologically to tumor cells.

SA is a residue on the extremes of glycoproteins and the sugar chain of glycolipids on cell membrane that is widely distributed on the surface of the cell membrane (including that of red cells) of mammals and is the main source of negative cellular electric charges. It is also an important component of receptors on cell membrane, taking part in physiological processes such as cell differentiation, the metastasis of cancer cells, and the recognition, adhesion, and tactile inhibition of cells. It is a key to determining what important features the cell membrane has[28,29]. Therefore, changes in the SA content of cell membrane can lead to a series of biological changes inside the cell. SA is also an important component of the C3b receptor (CR1) of red cells[30]. As mentioned earlier, CR1 is an important material basis for red cells to perform their immunological functions, that is to say, the amount of SA determines the activity of CR1 receptors, thus also determining the immune effect of the organism's red cells. Experimental results show that the SA content of red cell membrane of tumor-bearing mice has clearly decreased, and that it further decreases after chemotherapy. The SA content of red cell membrane of groups to which Haimiding was administered, on the other hand, was clearly higher than that of the negative control and the positive groups, and the gap between them was very large. Based on the above analysis, it seems that Haimiding serves to enhance the immunity of the organism by affecting the SA content in red cell membrane and thus the activity of CR1.

Since the SA content of cell membrane is closely related to its activity, further research on the effect of Haimiding on the activity of red cell membrane is very important. In isotonic solution, the membrane of red cells can reseal, thus restoring the permeability barrier to macromolecules and cations, forming a sealed membrane. The index measuring the capacity of red cell membrane to reseal is sealability. Due to the growth of the tumor and the effect of anti-tumor drugs, the functioning of the membrane will inevitably change, and sealability can be used to indicate the extent of this change. Our experimental results show that the sealability of the red cell membrane of tumor-bearing mice is lower than that of normal mice, but Haimiding can increase sealability, suggesting that it can help restore the functions of red cell membrane. 5-Fu, on the other hand, does not have this effect. Increase in the activity of red cell membrane results in its various functions approaching the normal, which is the fundamental reason why the immune efficacy of red cells is enhanced and why the secretion of SA is increased. The membrane of tumor cells can also reseal, but its sealability is lower than that of normal red cells. Findings from the treated groups show that both Haimiding and 5-Fu can lower the sealability of tumor cells, suggesting that the activity of cell membrane has been lowered, which may lead to the disorganization and death of the tumor cells.

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