

RAPID COMMUNICATION

Effects of body-resistance strengthening and tumor-suppressing granules on immune adhesion function of red blood cells and expression of metastasis protein CD44 in tumor cells of patients with esophageal carcinoma

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Abstract

AIM: To investigate the effects of Fuzheng Yiliu granules (body-resistance strengthening and tumor-suppressing granules) in patients with esophageal carcinoma.

METHODS: We compared the immune adherent properties of red blood cells (RBCs), the expression of metastasis protein CD44, and the metastasis inhibition factor nm23, in esophageal carcinoma tumor cells of patients before and after radiotherapy in the presence and absence of orally administered Fuzheng Yiliu granules. Sixty-three hospitalized patients with esophageal carcinoma were treated with standard radiotherapy and randomly divided into treatment group ($n = 30$) treated with both radiotherapy and Fuzheng Yiliu granules and control group ($n = 33$) given radiotherapy only. Blood samples and tumor tissue were obtained before and after 21 d of treatment. The rosette rates for complement receptor type 3b (C3bRR) and immune complex receptor (ICRR) on RBCs were measured by erythrocyte immunological methods. Expression of CD44 and nm23 in tumor tissue sections was determined by immunohistochemical staining with monoclonal antibodies CD44v6 and nm23H-1, respectively.

RESULTS: The positivity of RBC-C3bRR before and after 21 d of treatment increased from $7.78\% \pm 1.59\%$ to $10.03\% \pm 2.01\%$ in the double treatment group, while it changed only slightly from $7.18\% \pm 1.29\%$ to $7.46\% \pm 1.12\%$ in the radiotherapy group. The positive rate for RBC-ICRR decreased from $37.68\% \pm 2.51\%$ to $22.55\% \pm 1.65\%$ after the double treatment, and from

$37.28\% \pm 2.41\%$ to $24.69\% \pm 1.91\%$ in radiotherapy group at the same time points. The difference in erythrocyte immune adherent function between the two groups was significant ($P < 0.01$, *t*-test). The CD44⁺-cases were reduced from 21 (70.00%) to 12 (40.00%) after treatment with Fuzheng Yiliu granules, whereas the CD44⁺-cases (69.70%) in the radiotherapy group remained unchanged. The difference between the treatment (40.00%) and control (69.70%) groups was significant ($P < 0.05$). Although the nm23⁺-cases were increased from 4 (13.33%) to 6 (20.00%) in the double treatment group and from 6 (18.18%) to 7 (21.21%) in the radiotherapy group, the difference was not significant ($P > 0.05$).

CONCLUSION: Fuzheng Yiliu granules enhance the immune adhesion function of RBCs and reduce the number of CD44⁺-cells in esophageal carcinoma patients, suggesting a potential role of these Chinese herbals in suppression of invasion and metastasis of malignant cells. However, this anti-metastatic effect has yet to be validated *in vivo*.

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Key words: Fuzheng Yiliu granule; RBC immune function; CD44; nm23; Esophageal carcinoma; Metastasis; Randomized controlled clinical trial

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INTRODUCTION

Fuzheng-Yiliu granules (body-resistance strengthening and tumor-suppressing granules) were made from Gansu-produced herbals including *Radix hedysari*, *radix Radix angelicae sinensis*, *Rhizoma zekoariae* and *Radix patriniae* at a ratio of 3:1:1:3 by boiling, concentrating and adding an excipient. According to our clinical observations, the

combination of these four herbals at the ratios mentioned may suppress tumor cell growth and concomitantly enhance host immune function. Our previous study of Fuzheng Yiliu granules in an animal model indicated that Fuzheng Yiliu treatment inhibits S180 tumor cell growth in mice associated with suppression of telomerase activity and cell transition from G0/G1 to S phase^[1]. Our *in vivo* studies using tumor tissues derived from cancer patients treated with Fuzheng Yiliu granules have confirmed its suppressive role in cell proliferation and transition from G0/G1 to S phase, further demonstrating its ability to upregulate nuclear transcription factor NF κ B and apoptosis in breast and esophageal-gastric carcinomas^[2,3]. Very recently, we showed that Fuzheng Yiliu granules can lower the expression of metastasis protein CD46 in esophageal carcinoma^[4]. In this report, we describe the effect of Fuzheng Yiliu treatment on the immune adhesion function of red blood cells including RBC-C3bRR and RBC-ICRR as well as expression changes of the metastasis protein CD46 and the metastasis inhibition factor nm23 in tumor tissues.

MATERIALS AND METHODS

Patients

In this study, 63 in-hospital patients enrolled from Wuwei Oncology Hospital, Gansu Province, included patients with esophageal carcinoma diagnosed by esophageal-gastric endoscope and pathology on biopsy, patients who had no previous radiotherapy, and patients who received the complete treatment program. This study was approved by the Institutional Review Board of Wuwei Oncology Hospital.

Random groups

Sixty-three patients were divided into treatment and control groups using the randomization table. Patients (20 males and 10 females, ranging in age from 45 to 76 years with a mean age of 58.6 ± 7.9 years) in the treatment group were treated with both radiotherapy and Fuzheng Yiliu granules. Patients in the control group (22 males and 11 females, ranging in age from 47 to 70 years with a mean age of 57.3 ± 6.2 years) were treated with radiotherapy alone. These two groups were comparable and had no statistical differences ($P > 0.05$) in the case number, sex or age.

Clinical treatment

Patients in the control group were treated with the standard Co60 radiation at a dose of 1.8-2.0 Gy in 3 or 4 fields, 3 cm above and below the tumor lesions, 5 times a week for three weeks (total dose of 50-70 Gy). Patients in the treatment group were also given oral of Fuzheng Yiliu granules, 5.0 g each time, 3 times a day for 21 d. Fuzheng Yiliu granules were developed by the Institute of Combined Western and Chinese Traditional Medicine, Lanzhou University Medical School and manufactured by Lanzhou Foci Pharmacy Company. Five grams (5.0 g) of granules is equivalent to 45 g of original herbs.

Tumor tissue and blood samples

Upon admission to Wuwei Oncology Hospital, patients were subjected to electron esophago-gastroendoscopy for diagnosis and biopsies of 3-4 pieces of tumor tissue. After radiotherapy for both groups, in addition to oral Fuzheng Yiliu granules in the treatment group, the patients were again subjected to electron esophago-gastro endoscopy for diagnosis and biopsies of 3-4 pieces of tumor tissue. Tissues were formalin-fixed and paraffin-embedded for sectioning. Four microns of continuous sections was used for HE staining, pathological diagnosis and immunohistochemical staining. Two milliliters of blood samples drawn into anti-coagulation tubes from each patient before and after 21 d treatment was used to test immune function of red blood cells.

RBC-C3bRR and RBC-ICRR

Red cell immune adhesion function, RBC-C3bRR and RBC-ICRR, were detected as previously described^[5,6]. Yeast cells were obtained from the Blood and Immune Laboratory of Shanghai Hospital, the Second Military Medical University). If one red cell adhered to more than 2 yeast cells, it was counted as one rosette. The percentage of rosettes in 200 red cells was calculated.

Immunohistochemistry

Antibodies CD44v6 and nm23H-1 and related reagents were purchased from Boshide Company (Wuhan, China). Immunohistochemistry was performed by the avidin-biotin peroxidase complex (ABC) method (Boshide, Wuhan). Briefly, slides were deparaffinized, and rehydrated through xylene and alcohol in Coplin jars. Endogenous peroxidase was blocked with 3% H₂O₂ in phosphate-buffered saline (PBS) for 10 min, and tissues were washed 3 times in ddH₂O at room temperature. Microwave oven heat-induced epitope retrieval (HIER) was performed on sections used for CD44v6 antibody, and washed in PBS buffer. Tissue sections for nm23H-1 staining were treated with enzyme buffer from the kit and then washed with ddH₂O. The slides were incubated with normal goat serum for 20 min followed by primary antibody (rabbit monoclonal antibody CD44v6 or nm23H-1 with dilutions). Incubation was performed overnight at 4°C. After washed three times, slides were incubated with biotinylated goat anti-rabbit IgG at 31°C for 20 min. After washed three times, the slides were incubated in SABC solution. Slides were washed 4 times (5 min each time) and then developed with liquid DAB at 31°C for 20 min, washed twice with H₂O, and finally counterstained lightly with Mayer's hematoxyline for 5 s, dehydrated, cleared, and mounted with resinous mounting medium. CD44v6 stained brown-yellowish granules mainly on the cell membrane with some cytoplasmic staining. Nm23H-1 stained brown-yellowish granules in cytoplasm. Signal intensity and distribution were^[7-9] scored blindly by pathologists as previously described^[7-9]: distribution score (DS) was graded as 0: absent; 1: < 10%; 2: 10%-50%; 3: 51%-90%; or 4: > 90%. The intensity score (IS) was graded as IS0: no signal; IS1: weak; IS2: medium; or IS3: strong. The combined total score was determined as a total score (TS) = distribution

Table 1 Effect of Fuzheng Yiliu granules on RBC immune adhesion function (mean \pm SD, %)

Group	n	RBC-C3bRR		RBC-ICRR	
		Before treatment	After treatment	Before treatment	After treatment
Treatment	30	7.78 \pm 1.59	10.03 \pm 2.01 ^b	37.68 \pm 2.51	22.55 \pm 1.65 ^b
Control	33	7.18 \pm 1.29	7.46 \pm 1.12	37.28 \pm 2.41	24.69 \pm 1.91

^b $P < 0.01$ vs control group after treatment.

Table 2 Effect of Fuzheng Yiliu granules on CD44v6 and NM23H-1 expression (%)

Group	n	Treatment	CD44v6		NM23H-1	
			Positive	Negative	Positive	Negative
Treatment	30	Before	21 (70.00)	9 (30.00)	4 (13.33)	26 (86.67)
		After	12 (40.00) ^b	18 (60.00)	6 (20.00)	24 (80.00)
Control	33	Before	23 (69.70)	10 (30.30)	6 (18.18)	27 (81.82)
		After	23 (69.70)	10 (30.30)	7 (21.21)	26 (78.79)

^b $P < 0.01$ vs control group after treatment.

(DS) + intensity (IS) (TS0: sum 0; TS1: sum 1 to 3; TS2: sum 4 to 5; TS3: sum 6 to 7). TS0 and TS1 were considered negative.

Statistical analysis

Binomial distributions were used to compute P-values for positive and negative immunohistochemical staining of anti-CD44 or anti-nm23 antibodies on the tissue sections. SPSS 8.0, χ^2 and *t*-test were used to test the difference in P-values. $P < 0.05$ or $P < 0.01$ was considered statistically significant or very statistically significant, respectively.

RESULTS

The results of the immune adherent function of red blood cells before and after radiotherapy for patients with esophageal carcinomas with or without oral Fuzheng Yiliu granules are listed in Table 1. Prior to treatment, RBC-C3bRR and RBC-ICRR had no significant differences ($P > 0.05$). After 21 d of treatment, RBC-C3bRR increased from 7.78% to 10.03% and 7.18 to 7.46% in the treatment and control groups, respectively ($P < 0.01$). RBC-ICRR decreased significantly from 37.68% to 22.66% and 37.28% to 24.69% in the treatment and control groups, respectively ($P < 0.01$).

The immunohistochemistry results of metastasis protein CD44 and metastasis inhibition factor nm23 in esophageal carcinoma tumor cells of patients before and after radiotherapy, with or without oral Fuzheng-Yiliu granules are listed in Table 2. Prior to treatment, the difference in cases positively stained with CD44v6 or nm23H-1 antibodies between the treatment and control groups was not statistically significant ($P > 0.05$). The number of cases positively stained with CD44v6 decreased from 21 to 12 in the treatment group, whereas there was no change in the control group ($P < 0.05$). However, the

number of cases stained with nm23H-1 increased from 4 to 6 in the treatment group and 6 to 7 in the control group ($P > 0.05$).

DISCUSSION

Esophageal carcinoma occurs frequently in adult and elderly populations. According to traditional Chinese medicine, malignancy is referred to as dysphagia syndrome and its development and progression may be due to aging and deficiency in Qi and Yin or in Qi and blood, or stagnation of phlegm-dampness, heat-toxin accumulation, consumption of body fluid and blood resulted from unhealthy habit of food-intake and emotional upsets. Patients treated with radiotherapy often have invasive and metastatic tumors that may cause difficulty in swallowing or instant vomiting upon food intake, resulting in lack of nutrients to support physiological metabolism. Constant nutrient deficiency leads to catabolism of body materials, further decreasing the levels of Yang-Qi and healthy Qi, which in turn make stagnation of phlegm-dampness and heat-toxin accumulation more severe. In Fuzheng Yiliu granules, Radix hedysai enhances Qi, radix angelicae sinensis boosts blood production, Rhizoma zekoariae increases blood circulation to transport stasis, Radix patriniae promotes diuresis to remove damp and toxic metabolites from the body. These four herbs in combination augment healthy Qi and reduce pathogenic factors.

Our study indicates that the presence of abnormal immune adhesion function of red blood cells in patients with esophageal carcinoma, could decrease RBC-C3bRR and increase RBC-ICRR^[10]. RBC and WBC counts usually begin to decrease three weeks after radiotherapy, preventing patients from continuous treatment. It was reported that the rosette of tumor cells can adhere to RBC^[5], providing morphological support for the hypothesis that erythrocytes might play a role in prevention of tumor cells from metastasis. Gan and Zhang^[11] reported that increased RBC-C3bRR and decreased RBC-ICRR play an important role in tumor cell metastasis. These studies strongly suggest that improvement in immune adhesion function of RBC can suppress tumor cell metastasis^[12]. Radix angelicae sinensis and Rhizoma zekoariae increase blood microcirculation, modulate immune function, inhibit or kill tumor cells, while Radix patriniae enhances immune function and inhibits S180 tumor cell growth^[13]. All these data and the results of our studies on animal model and clinical patients^[1-4], demonstrate that Fuzheng Yiliu granules enhance immunity, suppress tumor cell proliferation and induce apoptosis of malignant cells.

CD44v6 is one of the alternative spliced variants of CD44^[14]. CD44 is an integral cell membrane glycoprotein that may play a role as cell surface adhesion molecules in lymphocyte migration to lymph nodes^[15]. Matsumura and Tarin^[16] first report that splice variants of CD44 may be associated with metastases and can be used in early diagnosis of cancer patients. Mayer *et al*^[17] found that expression of CD44 is associated with distant metastases, recurrence and increased mortality of gastric cancer. In the

field of esophageal carcinoma, many investigators reported that CD44v6 over-expression is significantly associated with tumor invasion, distant metastasis, and poor prognosis^[18-20]. Schmits *et al*^[21] showed that mice deficient in all known variants of CD44 develop granuloma in response to *Cryptosporidium parvum* infection. SV40-induced tumorigenicity of CD44-deficient fibroblasts can be suppressed by reintroduction of CD44 expression^[12]. These results suggest that over-expression of CD44 may be a consequence, rather than a cause, of malignant tumor progression. Further studies are needed to elucidate the molecular mechanisms underlying pharmacological function of Fuzheng Yiliu granules on CD44v6-positive cells.

NM23H-1 belongs to the NM23 gene family that has been shown to play a critical role in cellular proliferation, embryonic development, differentiation, oncogenesis, and tumor metastasis^[22]. Expression of NM23H-1 inversely correlates with metastatic potential and does not correlate to host immune response^[23]. MacDonald *et al*^[24] found that specific mutations in the human NM23 gene reduce the ability of the gene product to inhibit cell motility. Granzyme A (GZMA) induces a caspase-independent cell death pathway characterized by single-stranded DNA nicks and other features of apoptosis. Fan *et al*^[25] showed that NM23H1 is a GZMA-activated DNase that interacts with its specific inhibitor SET. After GZMA loading or cytotoxic T lymphocyte (CTL) attack, SET and NM23H1 translocate to nuclei and SET is degraded by GZMA cleavage, allowing NM23H1 to nick chromosomal DNA. Thus, loss of NM23H1 expression in tumors might make them resistant to immune surveillance by CTL and nature killer cells, since target cells with silenced NM23H1 are less sensitive to GZMA-induced apoptosis. The role of NM23H-1 in apoptosis is consistent with clinical observations that reduced expression of NM23H1 is associated with metastatic esophageal carcinoma^[26,27] and poor prognosis^[28, 29]. NM23H-1-positive cases increased from 4 to 6 in the treatment group, and from 7 in the control group (Table 1, $P > 0.05$), indicating that Fuzheng Yiliu granules have no effect on NM23H-1 expression. An alternative explanation is that the current sample size was too small to make a valid statistical analysis.

COMMENTS

Background

Many patients are diagnosed with esophageal carcinoma at intermediate or advanced stage, making surgical treatment impossible. Late diagnosis may also minimize other treatments such as radiotherapy. Radiotherapy for esophageal carcinoma is limited due to its side effects. Recently, we have used a Chinese herb to improve the efficacy, reduce the side effects of radiotherapy for esophageal carcinoma, and improve the patients' living quality and prognosis. However, the mechanism remains unknown.

Research frontiers

Chinese herbs used in anti-metastasis treatment of carcinoma are currently a hot spot in the area of natural anti-cancer agents.

Innovations and breakthroughs

The basic and prophase clinical trial confirmed that Fuzheng Yiliu granules can improve treatment efficacy for esophageal carcinoma. This article explores the effects of these herbs on esophageal carcinoma at cellular and molecular levels.

Applications

In order to keep the successive efficacy of Fuzheng Yiliu granules, these granules must be used for 21 d or more.

Terminology

Immune adhesion function of red blood cells (RBCs): RBCs are the most important eigen immunocell that has been identified. RBCs function in adhesion, concentration, killing antigen and cleaning immune complex in blood circulation, etc. Through the above functions, RBCs join the regulating immune functions of human body.

Peer review

This is a well-written manuscript describing the research on the anti-tumor effect of the herbal medicine. Fuzheng Yiliu granules enhance the immune adhesion function of RBCs and reduce the number of CD44+ cells in esophageal carcinoma, suggesting a potential role of these Chinese herbals in suppressing invasion and metastasis of malignant cells.

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