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EXPERIMENTAL STUDY

External use of Ruyanneixiao cream efficiently blocks precancerous mammary lesions by interfering with glycolysis induced by inhibition of hypoxia inducible factor-1α, hexokinase 2, phosphofructokinase, and pyruvate kinase M2 expression

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Abstract

OBJECTIVE: To investigate the effect of Ruyanneixiao cream (RYNX) on the expression of hypoxia inducible factor-1α (HIF-1α), hexokinase 2 (HK2), phosphofructokinase (PFK), and pyruvate kinase M2 (PKM2) mRNA and protein in MCF-10AT cells and in an animal model of precancerous mammary lesions.

METHODS: Following treatment of MCF-10AT cells with RYNX, tamoxifen (TAM) and YC-1 for 48 h, HIF-1α, HK2, PFK, PKM2 mRNA and protein expression was analyzed. Fifty female SD rats were randomly divided into control, model, TAM, and high- and low-dose RYNX groups, with 10 rats in each group. A precancerous mammary lesion model was established for all groups except the control group. High- and low-dose RYNX cream containing TAM was coated on the breasts of animals in the corresponding groups. The rat mammary tissue was removed in the 10th week and HIF-1α, HK2, PFK, PKM2 mRNA and protein was analyzed.

RESULTS: *In vitro* analyses demonstrated that, compared with the matrix group, HIF-1α, HK2, PFK, PKM2 mRNA and protein expression was significantly decreased in the RYNX group ($P < 0.05$). Compared with the YC-1 + RYNX group, HK2, PFK, and PKM2 protein expression was significantly reduced in the RYNX group. HIF-1α, HK2, PFK, and PKM2 protein expression was increased significantly in the model group ($P < 0.05$) compared with the control group, while HIF-1α, HK2, PFK, and PKM2 mRNA and protein expression was significantly decreased in both the high- and low-dose RYNX groups ($P < 0.05$), with the effect being greater in the high-dose group.

CONCLUSION: RYNX can block precancerous breast lesions by decreasing the expression of HK2, PFK, and PKM2 mRNA and protein via inhibition of HIF-1α mRNA and protein overexpression in a dose-dependent manner.

Keywords: Ruyanneixiao cream; Precancerous mammary lesions; Glycolysis; Hypoxia-inducible factor 1, alpha subunit; Hexokinase; Phosphofructokinases; Pyruvate kinase

INTRODUCTION

Breast cancer is a prevalent disease, and its incidence and mortality rate rank first and sixth, respectively, among female cancers.[1] It undergoes a multistep process as follows: normal tissue → hyperplasia → atypical hyperplasia → carcinoma in situ → infiltrating carcinoma. This development provides a theoretical basis for therapies that block/reverse precancerous lesions and reduce the incidence rate of breast cancer. The glycolysis of tumor cells preferentially occurs under aerobic conditions, a phenomenon known as the Warburg effect. It was shown that in the progression from normal breast tissue to infiltrating cancer, the more severe the breast lesions, the higher the glycolysis rate. Glycolysis is a undesirable prognostic factor.[2,3] A reduction in glycolytic levels in precancerous mammary lesions may result in inhibition of their progression to carcinoma. Hypoxia inducible factor-1α (HIF-1α) is an important link in tumor cell glycolysis, playing a crucial role in precancerous breast lesions and inducing a variety of proteins in the glycolysis pathway,[4] including three key enzymes: hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). Effective reduction of the amount and effect of these enzymes may significantly block glycolysis.[5]

Ruyanneixiao cream (RYNX) is a good medicine for external use in the treatment of hyperplasia of mammary glands and precancerous mammary lesions (national invention patent number: ZL201110029344.1). It was developed by combining a traditional external treatment with modern Traditional Chinese Medicine. It functions in breaking blood, promoting *Qi*, removing poison, warming channels, reducing phlegm and resolving masses, that mean it can relieve symptoms, restrain the damaged mammary gland, delay or reverse the pathological change. Previous studies showed that RYNX is effective in blocking precancerous mammary lesions.[6] However, its molecular mechanism of action remains unclear. In this study, we investigated whether hexokinase 2 (HK2), PFK, and pyruvate kinase M2 (PKM2) expression was decreased through inhibition of HIF-1α *in vivo* and *in vitro*.

MATERIALS AND METHODS

Drug preparation

The matrix was prepared as follows: 140 g stearic acid, 100 g Potassium hydroxide water solution and glycerinum were mixed, and then the appropriate amount of distilled water was added to a volume of 1000 mL.

RYNX was prepared using 6 g of each the following: Dingxiang (*Flos Syzygii Aromatici*), Moyao (*Myrrh*), Ruxiang (*Olibanum*) and Dahuang (*Radix Et Rhizoma Rhei Palmati*) 9 g respectively; Meiguihua (*Flos Rosae Rugosae*), Touguxiang (*Herba Gaultheriae Grenulatae*), Yanhusuo (*Rhizoma Corydalis Yanhusuo*), and Wangbuliuxing (*Semen Vaccariae Segetalis*). Then 3 g each of Shexiang (*Moschus*) and Bingpian (*Borneolum Syntheticum*) were added. The herbs were extracted by adding 75% ethanol. The different doses of drug extract were added to the matrix to obtain RYNX. High- or low-dose RYNX contained two or four times the concentration of the original drug per 1 g cream. In the same way, tamoxifen (TAM) was added to the matrix to generate TAM cream, and 1 g TAM cream contained 0.005 g tamoxifen.

RYNX transdermal liquid was administered by removal of the abdominal skin of rats, followed by lifting of the skin and administration of 2 g of high-dose RYNX. Samples was taken 24 h later, filtered using a 0.22 μm millipore filter, and preserved at 4 ℃.

Cell culture

MCF-10AT cell lines were purchased from the American Karmanos Cancer Institute. They were cultured in DMEM/F-12 medium supplemented with 5% horse serum and incubated at 37 ℃ in 5% CO_2. The intervention drug was added when the cells had reached approximately 50% confluency. The experiment was conducted after continuous culture for 48 h.

MTT assay

The effect of RYNX on MCF-10AT cell proliferation was detected by the MTT method. MCF-10AT cells at logarithmic growth phase were adjusted to 5×10^4 cells/mL and seeded into a 96-well culture plate. The cells were treated with culture solution containing different doses of RYNX transdermal liquid for 48 h. Then, 10 μL MTT (Sigma, St. Louis, MO, USA; 5 mg/mL) was added to each well and incubated for 4 h at 37 ℃. The supernatant was then discarded and 150 μL Dimethyl Sulphoxide (DMSO) was added to each well. Absorbance (A) was detected at 490 nm with a microplate reader and the cell inhibition rate was calculated.

Real-time PCR

Total RNA was extracted according to the TRIzon method. We did 2 step real time RT-PCR by Prime Script RT reagent kit (Takara, Shiga, Japan) and SYBR Premix EX Taq Ⅱ (Takara, Shiga, Japan). cDNA was synthesized according to the manufacturer's instruc-