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RESEARCH

Relationship Between Gastric Cancer Tau Protein Expression and Paclitaxel Sensitivity

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Abstract The abnormal expression of Tau protein in breast cancer tissue affects paclitaxel sensitivity. The abnormal expression also exists in gastric carcinoma. Therefore, we speculate that the expression levels of Tau protein is closely related to paclitaxel sensitivity in gastric cancer, thus affecting the efficacy of paclitaxel. In this study, we used immunohistochemical methods to detect Tau protein expression levels in 47 cases of gastric cancer specimens. We also used Western blot to detect the level of Tau protein expression in gastric cancer cell lines and to check the efficacy of paclitaxel in vitro application. Findings indicate that Tau protein expression rate can reach as high as (+ +–+ + +) 63.83 % in gastric cancer. Paclitaxel induces inhibition and apoptosis with low expression of Tau protein in gastric cancer cell lines ($P<0.05$). The level of Tau protein expression is significantly correlated with paclitaxel efficacy. If confirmed by further studies, the Tau protein can be another useful marker of gastric cancer, thereby leading to the application of paclitaxel in cancer treatment.

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Keywords Gastric cancer · *Tau* protein · Paclitaxel · Sensitivity

Introduction

Gastric cancer belongs to the group of malignant tumors with high incidence in our country. The combined treatment with chemotherapy is particularly important because the possibility for early diagnosis of gastric cancer is not high. However, there is no generally accepted standard for chemotherapy. Clinical treatment recommends therapies of fluorouracil and cisplatin as basic treatment and anthracycline or taxane treatment [1–3]. However, chemotherapy for advanced gastric cancer in remission is short, with a median survival of only 10 months or so. Frequent medicine resistance is the key cause of this problem, which influences further treatments. Therefore, in molecular biological target research in sensitive patients, overcoming and reversing taxane drug resistance is a major problem that needs to be resolved.

Paclitaxel is an anti-microtubule drug, whereas Tau protein is a microtubule-associated protein regulating tubulin dynamics. Tau protein is widely expressed in many tissues. Research on rats show that there is a high expression of this protein in the heart, lungs, skeletal muscle, kidney, and testes, but has a low expression in the adrenal gland, stomach, and liver. Different subtypes of Tau protein are present in different tissues. Some are low-molecular proteins, whereas others are high-molecular proteins [4–6]. In this study, we explore the relationship between the treatment of gastric cancer and paclitaxel resistance by detecting the expression of the Tau protein in gastric cancer tissues and the different reactions to taxol in vitro to provide a reference for clinical application.

Materials and Methods

Subjects

The study was conducted from January 2010 to October 2010 on surgical resection specimens of 47 cases of gastric cancer tissues in Jiangyin People's Hospital, Jiangsu province. All cases were pathologically diagnosed with gastric adenocarcinoma and had complete clinical data. The subjects consisted of 36 males and 8 females, aged 48 to 80 years (average of 66.2 years). All the patients did not undergo pre-operative radiotherapy and chemotherapy. The gastric cancer TNM staging used was based on the American Joint Commission on Cancer Standard (2007). This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Affiliated Jiangyin Hospital of Southeast University Medical College. Written informed consent was obtained from all participants.

Cell Cultivation

The human gastric cancer cell lines MKN45 and BGC823 were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

Immunohistochemistry Staining

The tissue samples embedded in paraffin wax were rehydrated. For antigen retrieval, 10 mM sodium citrate (pH6.0) was heated to approximately 95 °C, after which the tissue slide was heated in the solution for 15 min. The tissues were incubated in 3 % H_2O_2 under room temperature for 5 min to 10 min to eliminate endogenous peroxidase activity. The tissues were then treated with 5 % normal goat serum Phosphate buffered saline (PBS dilution) clone. The first antibody was incubated at 37 °C for 2 h or 4 °C overnight. Using the appropriate dilution ratio, biotin was added dropwise to mark the second antibody 1 % bovine serum albumin–Phosphate buffered saline (BSA–PBS dilution). The tissues were then incubated at 37 °C for 30 min. Horseradish peroxidase horseradish peroxidase (HRP)-labeled streptavidin working solution was added dropwise to the tissues, after which the tissues were incubated at 37 °C for 15 min. The tissues were stained using 3,3′-diaminobenzidine diaminobenzidine (DAB) for 3 min to 5 min (monitored under the microscope to avoid a strong color). Finally, the tissues were dehydrated in an alcohol gradient, mounted using neutral gum, and were observed and photographed under a microscope.

Tau Protein Expression Analysis

The Tau protein expression is indicated by the clear brown-yellow granules in the gastric cancer cell cytoplasm. Based on the extent and intensity of staining, cells were divided into four levels: (0) indicates no positive cells, no coloring (Fig. 1a); (+) indicates positive cells (<25 %), light yellow color (<25 %) (Fig. 1b); (++) indicates positive cells (25–50 %), brown-yellow color (Fig. 1c); and (+++) indicates positive cells (>50 %), dark brown color (Fig. 1d). Blind film-reading by three persons was performed on each slice. The average of the three results was used as the final expression.

Immunohistochemistry

MKN45 and BGC823 cells in logarithmic phase of growth were vaccinated in 24-well plates (5×10^4 cells/well). The first antibody (Tau46 Mouse mAb, 1:800 dilution) was incubated at 4 °C overnight. Plates were washed with PBS (pH7.2 to 7.6) for 2 min and repeated three times. Biotinylated goat anti-mouse IgG was added dropwise. Cells were then incubated at 20 °C to 37 °C for 20 min. Cells were washed with PBS (pH7.2 to 7.6) three times for 2 min. Straptived-HRP (SABC)was added to the cells, after which the cells were incubated at 20 °C to 37 °C for 20 min. PBS (pH7.2 to 7.6) washing was done to the cells for 5 min and repeated four times. One drop each of kit A, B, and C reagents were added to 1 mL of water to make the DAB stain. Stain was added to the slice after mixing, and then it was slightly stained with hematoxylin. The stained cells were observed under the microscope.

Western Blot

Cells were collected in a 1.5 mL centrifuge tube and washed with cold PBS twice. Cell lysate was added to the cells and was allowed to react for 1 min. The mixture was centrifuged for 5 min to collect the supernatant in a 1.5 mL centrifuge tube. Nano Drop 1000 was used to test the protein concentration. Buffer was then added and boiled in water for 5 min. For the sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)electrophoresis, 13 % SDS-PAGE was prepared. The polyvinylidene fluoride (PVDF) membrane was marked and placed in 1 % BSA (TBS preparation). The first antibody was combined with the antigen and incubated overnight at 4 °C inside a shaker. After discarding the first antibody, the shaker was cleaned with TBST for approximately 5 min while changing the medium repeatedly for 4 to 5 times. The second antibody was combined with the first antibody and incubated in the shaker at room temperature for 1 h. The second antibody was then discarded and the

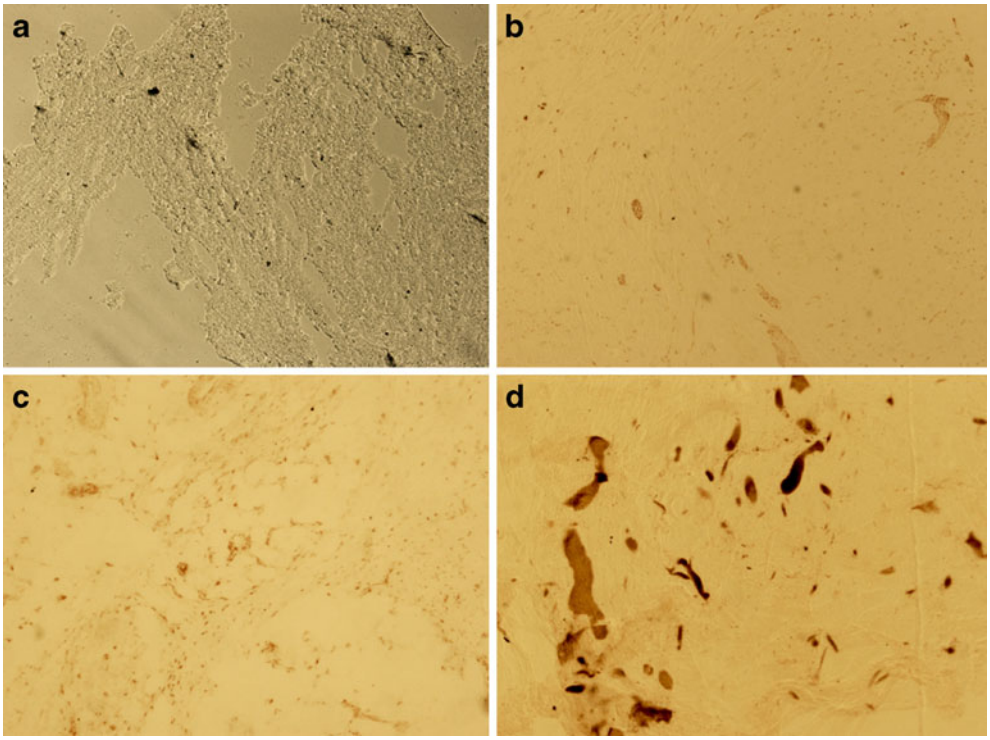

Fig. 1 Tau immunohistochemistry Staining Score Results of Gastric Cancer (SP×100). **a** Staining Assessment score 0; **b** Staining Assessment score +; **c** Staining Assessment score ++; **d** Staining Assessment score +++

shaker was cleaned with Tris-Buffered Saline Tween-20(TBST) (same as above). The second antibody was marked with Horseradish peroxidase (HRP) and compared using ECL for color exposure. Test results were then recorded. The X-ray film generated was dried, scanned, and saved.

Cytotoxicity Analysis

MTT analysis of paclitaxel in gastric cancer cells MKN45 and BGC823 was conducted to test for cytotoxicity. Logarithmic phase cells and vaccines were placed in 96-well plates, with three re-holes in each set. Cells were starved overnight while adding saline or 6, 8, 10, 30, 50, 70, and 90 μg/mL of taxol. Cultivation was continued for 24 h. Up to 10 μL of 5 mg/mL of MTT was added to each hole. Cells were then incubated at 37 °C for 4 h. The supernatant was taken out and 100 μL Dimethyl sulfoxide (DMSO) was added to each hole, shock saluting the crystallization at normal room temperature for 10 min. Absorption bare-degree value was measured at 570 nm (absorption wavelength) and 630 nm (reference wavelength). The inhibition rate was then calculated using the following formula:

$$\text{Cell inhibition} = [1 - \text{Experimental group}(OD_{570\,\text{nm}} - OD_{630\,\text{nm}})/\text{the control group}(OD_{570\,\text{nm}} - OD_{630\,\text{nm}})] \times 100\%$$

Flow Cytometry

Annexin Fluorescein isothiocyanate (V-FITC)/propidium iodide (PI) double staining was used for apoptosis detection. Adherent cells were collected for trypsin digestion without Ethylenediaminetetraacetic acid (EDTA). Cells were cleaned twice with BS, after which the 5×10^5 cells collected were added with 500 μL binding buffer suspension cells and 5 μL annexin V-FITC. After mixing, 5 μL PI was added to the solution and re-mixed. Detection with flow cytometry was conducted using the stimulating wavelength Ex = 488 nm and emitting wavelength Em = 530 nm. Normal cells without apoptosis-inducing treatment were used as reference for fluorescence compensation adjustment to remove spectral overlap. In addition, the position of cross gate was also set.

Statistical Analysis

Using SPSS 15.0 software for statistical analysis, data were compared using x^2 test method. Groups were compared using repeated measures of ANOVA. $P<0.05$ indicated statistical significance.

Results

Tau Protein Expression

Tau protein expression is observed in the cytoplasm. The staining assessment shows that 3+ – 2+ is positive and 1+ – 0 is negative (Fig. 1). Tau protein (3+) expression is equivalent to 12.77 % (6/47), (2+) expression is equivalent to 51.06 % (24/47), and (1+) expression is equivalent to 38.30 % (18/47). The overall positive rate of the 47 cases of gastric cancer is 63.83 % (Fig. 2).

Western Blot

Western blot was used to detect the level of Tau protein expression in the gastric cancer cell lines. Tau protein expression in MKN45 gastric cancer cell line is relatively high. By contrast, the expression is relatively low in the BGC823 gastric cancer cell line (Fig. 3).

Growth Inhibition

MTT cytotoxicity analysis for paclitaxel in gastric cancer cells MKN45 and BGC823 shows that paclitaxel inhibits both cell lines. However, the cell function for the low expression of Tau protein in BGC823 cells is more obvious ($P<0.05$) (Fig. 4).

Apoptosis

The results of apoptosis detection in the MKN45 and BGC823 cell lines using flow cytometry indicate that paclitaxel induces apoptosis for both cell lines. The apoptosis rate of the low Tau protein expression for the BGC823 cell lines is more significant ($P<0.05$) (Fig. 5).

Discussion

Taxane has a broader application in current chemotherapy regimens for advanced gastric cancer due to its strong antitumor activity and relatively low toxicity. A number of clinical studies and randomized multi-center Phase III clinical trials (V325) support a joint program with taxane treatment for advanced gastric cancer, which shows very good results [7]. However, the frequent occurrence of drug resistance to the treatment of advanced gastric cancer seems to be a bottleneck. Therefore, to explore the molecular biological targets for the sensitive populations, there is an urgent need to address the major problem of overcoming and reversing taxane resistance in the field of cancer chemotherapy.

Paclitaxel is classic type of anti-microtubule drug whose mechanism is different from other anti-microtubule drugs, such as colchicine and vinca alkaloids. The latter mainly causes microtubule disassemblage and microtubule breakdown. Paclitaxel is typical stabilizer for microtubule that can promote the assembling and stabilizing for the microtubule specifically bind to the β position on the small tube, resulting in microtubule polymerization into clumps and bundles, leading to stability. These effects can inhibit normal microtubule network reorganization [8].

Based on a previous study, the mechanism of paclitaxel resistance is mainly due to the increase in P-glycoprotein (P-

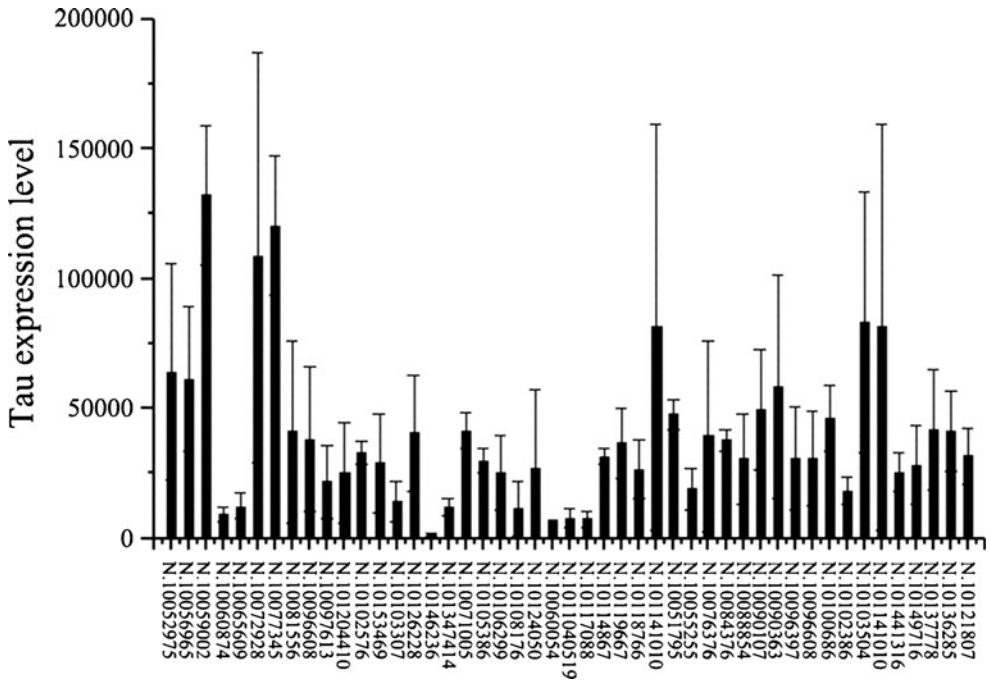

Fig. 2 Expression of Tau-protein in 47 cases Gastric Cancer

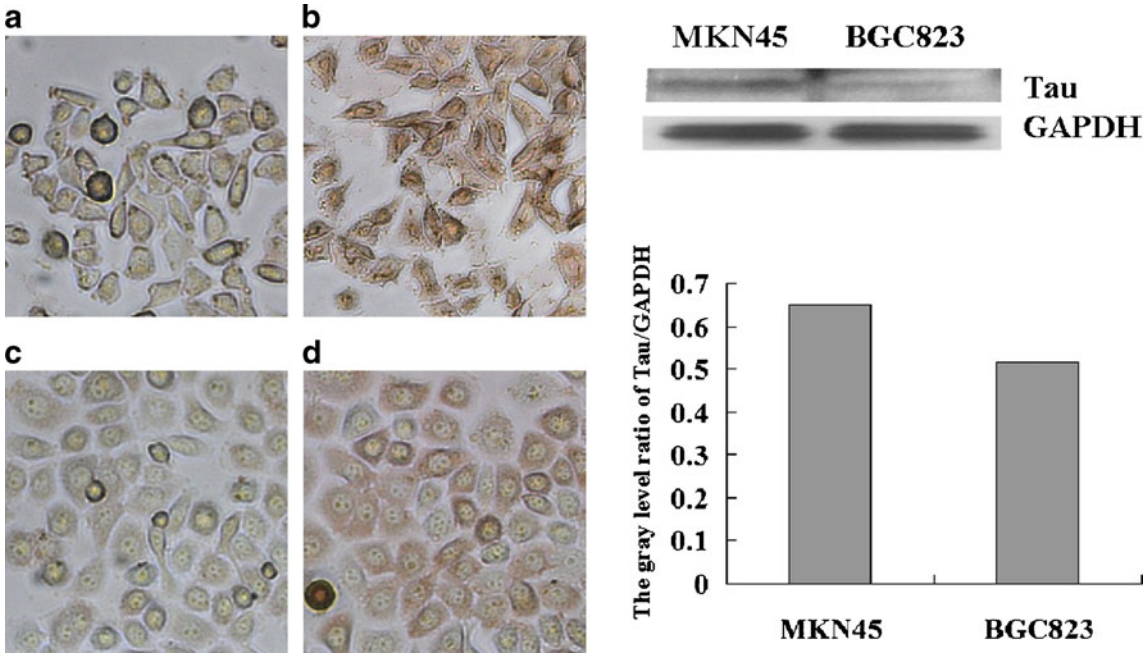

Fig. 3 The Expression Level of Tau-Protein in MKN45 and BGC823 Cell Lines (SABC×100). I: Immunohistochemistry detection expression levels of Tau of MKN45, BGC823 cell line. **a** MKN45 cell line control group (not the first antibody, only the second antibody); **b** MKN45 cell line Tau immunohistochemistry staining; **c** BGC823 cell line control group (not the first antibody, only the second antibody); **d** BGC823 cell line Tau immunohistochemistry staining. II, III: Western blotting detecting expression level of Tau in MKN45, BGC823 cell line, *$P<0.05$

glycoprotein, P-gp) expression, microtubule; and α and β subunit changes; and PKC, bcl-2, p53, and ErbB2 expression changes in the apoptotic pathways [9].

Tau protein is a microtubule-associated protein that promotes cytoskeletal microtubule polymerization and stability and regulates tubulin dynamics. The gene located on chromosome 17q21 contains 16 exons. Tau protein is mainly expressed in neurons and glial cells, regulating nerve cell growth and development. It plays an important role in nervous system formation and axon communication (conduction). However, Tau protein expression in other parts of the body is low. The abnormal expression of Tau protein was previously reported in Alzheimer's disease. According to previous literature, detection of the specific Tau protein with abnormal phosphorylation in the cerebrospinal fluid and the monoclonal antibody of the specific Tau protein may provide an effective means for predicting Alzheimer's disease [10]. In addition Tau protein has a function in chromosomal stability to a certain extent [11].

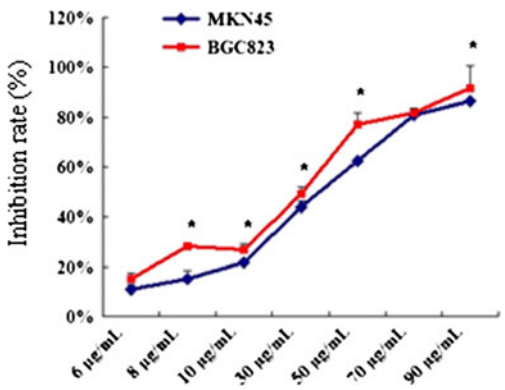

Fig. 4 MTT Analysis of Growth Inhibition on Paclitaxel to MKN45, BGC823 cell lines

Some researchers conducted a study to determine the relationship of Tau protein expression with efficacy of paclitaxel in breast cancer treatment. They found that the high expression of Tau protein is related to paclitaxel resistance in breast tissues in the treatment of breast cancer patients undergoing the taxane program. Immunohistochemistry showed that after remission was completed, 70 % of the patients' tumor tissues did not express Tau protein. In a follow-up study, the risk recurred for the patients who were positive with Tau protein (HR=0.50, 95 % CI, 0.32–0.78, P=0.002). Risk of death (HR=0.49, 95 % CI, 0.29–0.83, P=0.008) was significantly reduced. These results show that Tau protein-positive is an independent prognostic factor for breast cancer patients [12, 13]. However, among patients with breasts cancer and high Tau protein expression undergoing tamoxifen treatment, the no-recurrence time is significantly prolonged. This finding is a valuable endocrine therapy predictor. Therefore, for ER-positive breast cancers, the high Tau expression shows sensitivity to endocrine treatment but resistance to paclitaxel combined chemotherapy.

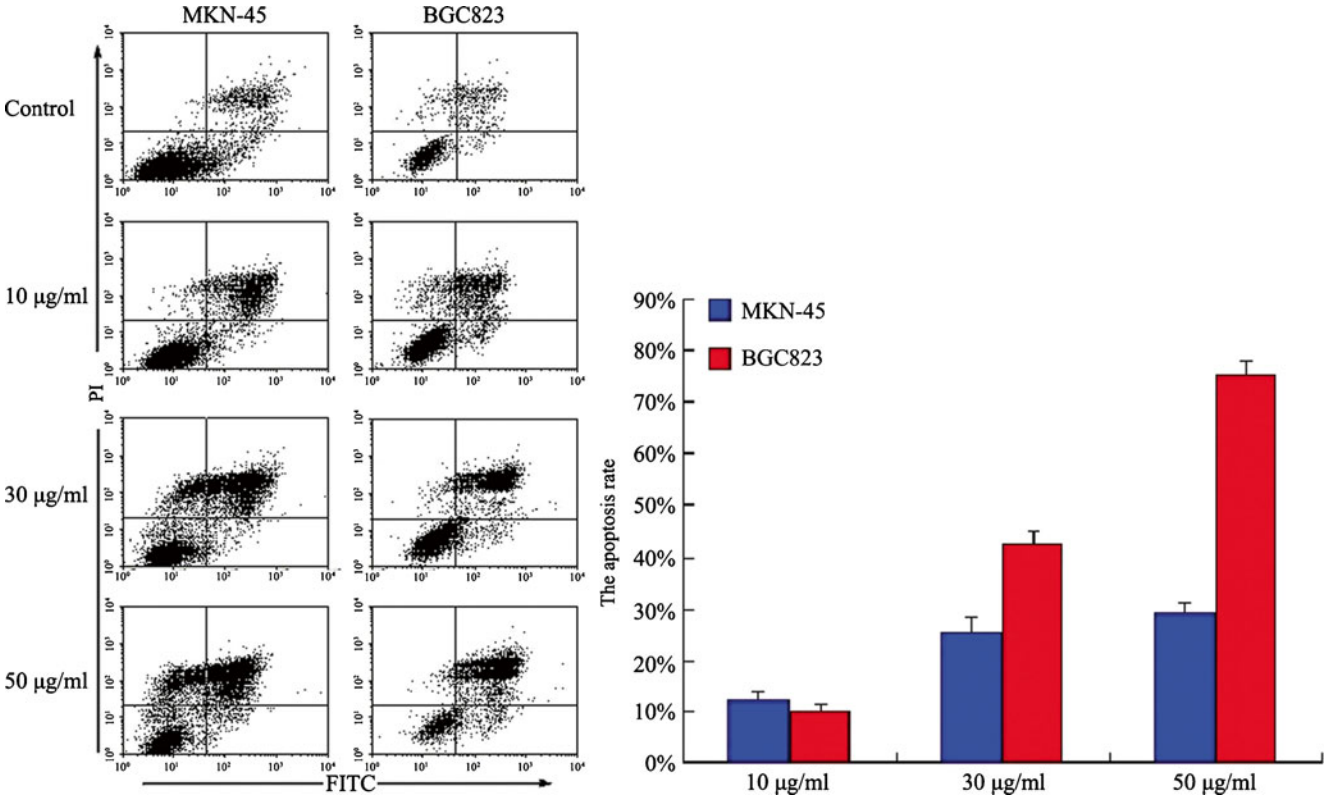

Fig. 5 Flow cytometry of induced apoptosis on paclitaxel to MKN45, BGC823 cell lines. **a** The apoptosis of different doses of paclitaxel on two groups of cell strains; **b** The comparison on apoptosis of different doses of paclitaxel on two groups of cell strains

Otherwise, in case there is low Tau expression, patients will not be sensitive to endocrine therapy but will benefit from the paclitaxel combined chemotherapy [14, 15].

Rouzier et al. found that multidrug-resistant tumor cells exhibit increased sensitivity only to paclitaxel after RNA interference in vitro experiments [16], wherein they reduced mRNA expression of Tau protein in breast cancer. The results indicated that paclitaxel exhibits better efficacy in patients with low Tau protein expression.

Local and international reports on Tau protein in gastric cancer have been conducted [17]. Mimor et al. reported [18] 20 cases of gastric cancer, among which 14 (70 %) were Tau protein positive (+++–++) and 6 (30 %) were negative (+–0). The six cases with negative expression responded to paclitaxel treatment, whereas 12 of the14 positive cases did not respond to paclitaxel treatment. This finding indicates that Tau protein levels are significantly associated with the efficacy of paclitaxel. Ren Feng et al. [19] also found that paclitaxel has a stronger inhibition and proliferation to gastric cancer cells of low Tau protein expression, and that apoptosis can also be enhanced. Qiu et al. [20] confirmed that low Tau expression is more sensitive to paclitaxel.

In the current study, we found that Tau protein has high expression in gastric cancer tissues. The positive rate of 63.83 % is very close to the value presented in previous reports [18]. Paclitaxel has a more obvious function to cells with low expression of Tau protein; the apoptosis rate is also higher. Paclitaxel may be easily combined with tubulin under low concentrations of Tau protein. The high concentration has a stabilizing effect to microtubules, reducing the harmful effects of paclitaxel, which leads to drug resistance. However, resistance mechanisms to paclitaxel and their associated prognostic value needs further study.

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