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The role of 5-fluorouracil in Wnt/β-catenin signalling in human papillomavirus-positive cervical cancer cells.

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Keywords: 5-fluorouracil; high-risk HPV-positive cervical cancer cells; T lymphocytes; Wnt/β-catenin signalling pathway; apoptosis.

Abstract. Human papillomavirus (HPV) infection is a major risk factor for cervical cancer, especially persistent infection with high-risk HPV. 5-fluorouracil (5-FU) is a widely used antimetabolite chemotherapeutic agent that inhibits the proliferation of tumour cells by interfering with ribonucleic acid and deoxyribonucleic acid synthesis; however, its mechanism of action has not been fully elucidated. This study aimed to investigate the role of Wnt/β-catenin signalling in patients with high-risk HPV with cervical cancer treated with 5-FU. Patients with high-risk HPV-positive cervical cancer treated with surgery were taken as the research participants, and lesion tissues were collected during surgery. Human HPV-positive cervical cancer cells were isolated and cultured in vitro by the enzyme combined digestion method, and the obtained cells were divided into a control group, a paclitaxel group and a 5-FU group. A 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to measure the proliferation of high-risk HPV-positive cervical cancer cells under different treatment conditions. Western blotting was used to evaluate the protein expression level of the Wnt/β-catenin signalling pathway in cells, and flow cytometry was used to analyse the level of T lymphocytes in the patients' blood. The results of the MTT assay showed that the proliferation rate of cervical cancer cells in the control group was significantly higher than that in the paclitaxel group and the 5-FU group at all detection time points ($p<0.05$). The expression levels of Wnt/β-catenin protein in the 5-FU group were lower than those in the paclitaxel and the control groups ($p<0.05$). The results of the T lymphocyte level comparison showed that the ratios of $CD3^+$ T cells, $CD4^+$ T cells and $CD4^+/CD8^+$ cells affected by 5-FU were higher than those before treatment ($p<0.05$). 5-fluorouracil can significantly reduce the expression level of Wnt/β-catenin protein and increase the T lymphocyte levels in cervical cancer cells.

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Función del 5-fluorouracilo en la señalización Wnt/β-catenina en células de cáncer de cuello uterino positivas al virus del papiloma humano.

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Palabras clave: 5-fluorouracilo; células de cáncer de cuello uterino positivas al VPH de alto riesgo; linfocitos T; vía de señalización Wnt/β-catenina; apoptosis.

Resumen. La infección por el virus del papiloma humano (HPV) es un factor de riesgo importante para el cáncer de cuello uterino, especialmente la infección persistente con HPV de alto riesgo. El 5-fluorouracilo (5-FU) es un agente quimioterapéutico antimetabólico ampliamente utilizado que inhibe la proliferación de las células tumorales al interferir con la síntesis de ARN y ADN, pero su mecanismo de acción no ha sido completamente elucidado. Para investigar el papel de la señalización Wnt/β-catenina en los pacientes con cáncer de cuello uterino de alto riesgo HPV tratados con 5-FU. Los pacientes con cáncer de cuello uterino positivo para HPV de alto riesgo tratados con cirugía fueron tomados como objetos de investigación, y se recolectaron tejidos lesionales durante la cirugía. Se aislaron y cultivaron in vitro células de cáncer de cuello uterino positivas para HPV humano mediante el método de digestión combinada enzimática, y las células obtenidas se dividieron en un grupo de control, un grupo de paclitaxel y un grupo de 5-fluorouracilo. El MTT se utilizó para medir la proliferación de las células de cáncer de cuello uterino positivas para HPV de alto riesgo bajo diferentes condiciones de tratamiento. La técnica de western blot se utilizó para evaluar el nivel de expresión proteica de la vía de señalización Wnt/β-catenina en las células. La citometría de flujo se utilizó para analizar el nivel de linfocitos T en la sangre del paciente. Los resultados del ensayo MTT mostraron que la tasa de proliferación de las células de cáncer de cuello uterino en el grupo de control fue significativamente mayor que en el grupo de paclitaxel y el grupo de 5-fluorouracilo en todos los puntos de detección ($p<0,05$). Los niveles de expresión de la proteína Wnt/β-catenina en el grupo de 5-fluorouracilo fueron inferiores a los del grupo de paclitaxel y el grupo de control ($p<0,05$). Los resultados de la comparación del nivel de linfocitos T mostraron que las proporciones de células T CD3+, células T CD4+ y células CD4+/CD8+ afectadas por el 5-fluorouracilo fueron más altas que antes del tratamiento ($p<0,05$). El 5-fluorouracilo puede reducir significativamente el nivel de expresión de la proteína Wnt/β-catenina y aumentar la actividad del nivel de linfocitos T en las células de cáncer de cuello uterino.

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INTRODUCTION

Cervical cancer is a common type of malignant tumour in women worldwide and has become a major disease that affects and threatens the survival status of women in various countries. The incidence of cervical cancer in China has been rising in recent years, with approximately 500,000 new cases every year [1]. There are many factors in

the development of cervical cancer, among which human papillomavirus (HPV) infection is the main risk factor; in particular, persistent infections of high-risk HPV present a higher risk of cervical cancer [2]. Although the popularisation of the HPV vaccine and the implementation of cervical cancer screening programmes have reduced the incidence of cervical cancer to a certain extent, determining how to effectively treat HPV-positive patients or patients with cervical cancer that has progressed to an advanced stage is still a major problem for doctors [3].

As a widely used antimetabolite chemotherapy drug, 5-fluorouracil (5-FU) has been extensively used in the treatment of a variety of solid tumours. It inhibits swelling by interfering with ribonucleic acid and deoxyribonucleic acid synthesis during the proliferation of tumour cells; however, its mechanism of action has not been fully elucidated. Studies have shown [4] that 5-FU must be maintained within a certain concentration in cervical cancer, and the appropriate extension of the administration time can allow more cells to be exposed to the drugs, which helps to increase the sensitivity of the drug. In recent years, some scholars have pointed out that 5-FU may exert its anti-tumour effect by affecting signalling pathways in the tumour microenvironment, such as the Wnt/β-catenin signalling pathway. The Wnt/β-catenin signalling pathway is a key intracellular signalling network that plays a central role in regulating cell fate, maintaining tissue homeostasis and tumour development [5]. In many tumour types, aberrant activation of the Wnt/β-catenin signalling pathway is strongly associated with tumour aggressiveness, drug resistance and poor prognosis. However, research on the Wnt/β-catenin signalling pathway has focused more on colon cancer and oral cancer, and few studies are related to high-risk HPV-positive cervical cancer.

In addition, immune cells in the tumour microenvironment, especially T lymphocytes, have an important impact on tumour development and response to treatment. T lymphocytes play an immune surveillance role by recognising tumour antigens, and in some cases, they can interact with tumour cells and influence apoptosis [6]. However, the interaction between T lymphocytes and the Wnt/β-catenin signalling pathway in HPV-positive cervical cancer and its role in the treatment of 5-FU is unclear [7,8].

Given this, the mechanism of action of 5-FU in the treatment of high-risk HPV-positive cervical cancer was analysed, and the apoptosis effect of the Wnt/β-catenin signalling pathway on tumour cells and their interaction with T lymphocytes in the process of promoting apoptosis is discussed.

PATIENTS AND METHODS

Patients with high-risk HPV who underwent surgery in our hospital between June 2022 and December 2023 were selected for the experiment. A total of 78 patients with positive cervical cancer were enrolled in the study. All patients' surgeries were performed by the same team of doctors. The study was approved by the ethics committee of the hospital, and the patients and their families voluntarily signed informed consent forms; the whole experiment lasted for 2 weeks. The **inclusion criteria** were as follows: (1) patients who met the diagnostic criteria for cervical cancer and had a histopathologically confirmed diagnosis of high-risk HPV-positive cervical cancer [9]; (2) patients with no serious heart, lung, kidney or other vital organ dysfunction and who were able to tolerate surgery and follow-up treatment; (3) patients who had not received radiotherapy or chemotherapy within 3 months before and after enrolment; (4) patients who could undergo surgical treatment; (5) patients whose examination and treatment was completed under the guidance of doctors. The **exclusion criteria** were as follows: (1) patients with a history of severe allergies, especially those who were allergic to 5-FU or its adjuvant drugs; (2) pregnant or lactating

women; (3) patients with a history of other malignant tumours or who had other malignant tumours at the same time; (4) patients with a history of mental illness or cognitive dysfunction and could not cooperate with the completion of surgery and return visits; (5) patients with serious infection or immunodeficiency disease, which affected the observation of the condition and the evaluation of treatment effect; (6) patients with severe coagulation dysfunction or bleeding tendency; (7) patients who successfully completed the operation but did not cooperate with the return visits.

Main instruments and reagents

The key chemicals and laboratory equipment used in the experiments were as follows: 5-FU (Hubei Hengjingrui Chemical) and paclitaxel as chemotherapy drugs for specific biological effects; type I collagenase, which is used in the isolation process of tissues or cells; Dulbecco's modified Eagle's medium (DMEM) serves as a substrate for cell culture, providing essential nutrients [10,11]. Routine laboratory equipment – centrifuges for sample separation; fully automated enzyme label analyser for rapid and accurate determination of enzyme-linked immunosorbent assay (Nanjing Nuovezan Biotechnology, Nanjing, China); CO_2 cell culture incubator (Shanghai Jinghong Experimental Equipment Co., Ltd, Shanghai, China) to provide a suitable growth environment for cells; and a C-MAGhS10 magnetic stirrer for mixing operations during experiments.

Culture of lesion cells

The following is a description of the procedure that was adopted and the equipment used. In a sterile environment, take a small piece (volume 0.5–1 cm^3) of surgically excised tumour tissue and place it in a file containing 10% foetal bovine serum (FBS) (Guangzhou Ruite Biotechnology) with DMEM (Zhejiang Senrui Biotechnology). Rinse thoroughly three times with phosphate-buffered saline (PBS) (Zhejiang Senrui Biotechnology) to remove impurities, then briefly soak in a solution containing penicillin and streptomycin to sterilise, then rinse again with PBS. Use ophthalmic surgical scissors to cut the tissue into fine fragments (approximately 0.5 × 0.5 mm per piece). Subsequently, add 0.25% trypsin solution (Zhejiang Senrui Biotechnology) to the tissue and digest at 37°C in a 5% CO_2 incubator for 30 min, until the tissue is soft. After digestion, remove the upper layer of liquid, then add 3 ml of 0.2% collagenase type I (Zhejiang Senrui Biotechnology) and continue digestion under the same conditions for 30–60 min to ensure complete tissue breakdown. Neutralisation is performed using DMEM with 10% FBS and filtered through a 200 mesh sieve. The collected cells are further ground through a 10 ml syringe core tube to form a single-cell suspension. After 5 min of centrifugation (at a force of 1,105 g), remove the supernatant and then resuspend the cells in DMEM containing 15% FBS, adjusting the cell concentration to 1×10^6 cells per mL. Cells are seeded in 30 cm^2 flasks and cultured at 37°C and 5% CO_2. After 24 h, observe cell attachment using an inverted microscope (Thermo Fisher), change the medium after 48 h to remove unattached cells and change the medium every 72 h. When cell confluency coverage reaches 80%–90%, digestion is performed using 0.25% trypsin, and subculture is performed according to cell density. After the culture is complete, cell identification is performed to ensure that the obtained cells are high-risk HPV-positive cervical cancer cells [12,13]. Cells are cultured on microscope slides and morphological characteristics of the cells are observed using light microscopy. Cervical carcinoma cells usually present as epithelioid cells with irregular nuclei and abundant cytoplasm.

Handling of cells

Cervical cancer cells cultured to the third generation were randomly assigned to three experimental groups: a control group,

a paclitaxel group and a 5-FU group. Control group: cells were not treated with any drugs and were maintained under basal culture conditions. Paclitaxel group: cells were co-cultured with 0.7 μmol/L of paclitaxel during culture. 5-fluorouracil group: cells were co-cultured with 9.6 μmol/L of 5-FU. The number of cells in each group was consistent, and three replicate wells were set up in each well to ensure the reliability of the experimental results.

Cell proliferation rate

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to measure the proliferation of high-risk HPV-positive cervical cancer cells under different treatment conditions [5]. The experiment terminated the culture at different time points and completed the determination of the cell proliferation rate. At the end, 20 μg of MTT solution (5 mg/mL concentration) was added to each sample well and incubated at 37°C for 4 h, followed by removal of the medium. Approximately 150 μLof dimethyl sulfoxide was added and shaken at low speed for 10 min on a shaker to dissolve the previously formed formalised methazole blue crystals; finally, the absorbance of each well was measured at a wavelength of 492 nm.

Wnt/β-Catenin protein expression detection

Western blot was used to evaluate the protein expression level of the Wnt/β-catenin signalling pathway in cells [6]. The three groups of cells were added to a RIPA lysis buffer, and lysis was performed on ice to ensure complete rupture of the cell membrane and release of intracellular proteins. Cell debris was removed by centrifugation (12,000 rpm, 10 min) and the supernatant was collected to obtain protein samples. Extracted proteins were quantified using either the bicinchoninic acid assay method or the Bradford method, ensuring that the same amount of protein was loaded in each sample for comparison. Quantified protein samples were mixed with loading buffer and heated at 95°C for 5 min to denature. Samples were loaded into polyacrylamide gels and subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Depending on the molecular weight of the protein, the appropriate concentration of gel (typically 10%–15%) was selected. Following the completion of electrophoresis, proteins from the gel were transferred onto polyvinylidene difluoride (PVDF) membranes. Following membrane transfer, the PVDF membranes were blocked with 5% non-fat dry milk and incubated for 1 h at room temperature to prevent nonspecific binding. The membranes were incubated with specific primary antibodies and incubated overnight at 4°C. The following day, the membranes were washed to remove unbound primary antibodies and washed three times for 5 min each using tris-buffered saline with 0.1% Tween® 20 detergent buffer. The membranes were incubated with horseradish peroxidase (HRP)-labelled secondary antibodies for 1 h at room temperature. The membranes were washed again to remove unbound secondary antibodies. The membranes were processed using a chemiluminescent substrate, and HRP reacted with the substrate to produce a detectable luminescent signal. Signals were captured using an imaging system and the images were recorded. Finally, the Wnt/β-catenin was developed in the gel imaging system β-actin bands and corresponding grey values, and the expression level of each group was calculated, with β-actin as the internal control.

T lymphocyte level

A 4 mL sample of fasting venous blood was drawn from patients with cervical cancer before and after surgery. The principle of aseptic operation during collection was strictly followed to avoid sample contamination. The collected blood sample was mixed with an appropriate amount of anticoagulant (heparin) to prevent blood coagulation and ensure the smooth progress of the subsequent separation process. The blood sam-

ple was centrifuged at a relative centrifugal force of 1,180 g for 15 min. During centrifugation, lymphocytes with lower density float in the upper layer of the density gradient medium, while other components with higher density, such as red blood cells and white blood cells, sink in the lower layer. At the end of centrifugation, the lymphocyte layer located in the upper layer was carefully collected to avoid disturbing other components of the lower layer. Lymphocytes were gently aspirated using a pipette and transferred to a new centrifuge tube. Collected lymphocytes were washed with sterile PBS buffer to remove residual density gradient media and other impurities. After washing, centrifugation was performed again, the supernatant was discarded and the precipitated lymphocytes were retained. The $CD3^+$, $CD4^+$, $CD8^+$ cells and the $CD4^+/CD8^+$ ratio in blood were analysed by flow cytometry (Beijing Boao Jingdian Biotechnology Co., Ltd.) [15,16].

Experimental data processing and analysis

The SPSS software package was used for statistical analysis. For the measurement data that met the normal distribution condition, the independent samples *t*-test and one-way analysis of variance method were used, and the results obtained were measured as the mean ± standard error. For categorical data, the chi-squared test (χ^2) was used for analysis. A *p*-value of <0.05 was used to indicate that the difference between the data is statistically significant.

RESULTS

Comparison of cell proliferation rates at different time points between the three groups of cells

The MTT assay showed that the proliferation rate of cervical cancer cells in the paclitaxel group and the 5-FU group was significantly lower than that in the control group ($p<0.05$) at all detection time points, indicating that the paclitaxel level was significantly lower than that of the control group ($p<0.05$). Both drug treatments with fluorouracil can effectively inhibit the proliferation ability of cervical cancer cells. In addition, the cell proliferation rate of the 5-FU group was lower than that of the paclitaxel group at all time points ($p<0.05$), indicating that 5-FU had a stronger inhibitory effect on cell proliferation (Fig. 1).

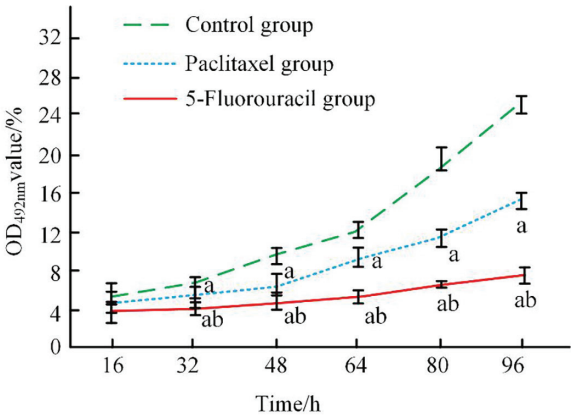

Fig. 1. Comparison of cell proliferation rates at different times. $p<0.01$ between different groups, one-way ANOVA was used for statistical analysis.

Comparison of Wnt/β-catenin in different organisations

The protein expression levels of Wnt/β-catenin in the 5-FU group and paclitaxel group were lower than those in the control group ($p<0.01$). The expression levels of Wnt/β-catenin protein in the 5-FU group were lower than those in the paclitaxel group ($p<0.01$) (Fig. 2).

Comparison of T lymphocyte levels before and after surgery in the observation group

The levels of $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+/CD8^+$ ratio were (68.94 ± 3.58), (43.28 ± 2.14), (24.17 ± 2.11) and (2.02 ± 1.00) in the patient after 5-FU treatment, respectively. The level of all cells was significantly higher than that before 5-FU treatment ($p<0.05$). This reflects the activation and enhancement of the intracellular immune response after treatment.

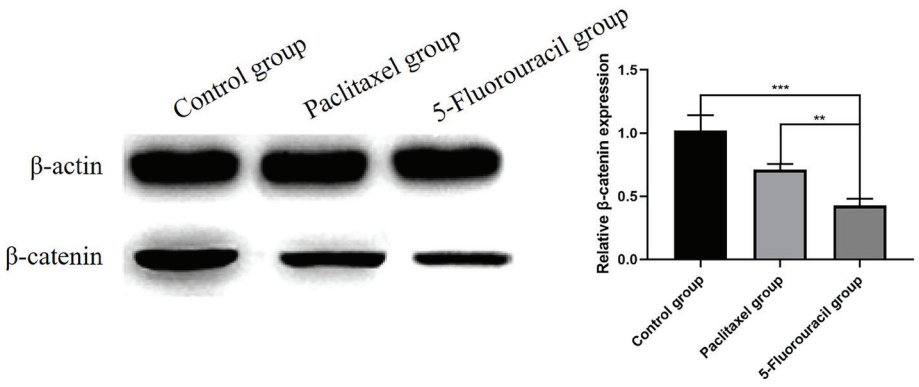

Fig. 2. Comparison of protein expression of cellsWnt/β-catenin in three groups. **: p<0.01, ***: p<0.001, one-way ANOVA was used for statistical analysis.

An increase in the $CD4^+/CD8^+$ ratio indicates an increase in helper T cells relative to cytotoxic T cells. This involves a more effective immune response. In contrast, the level of $CD8^+$ T cells (cytotoxic T cells) in patients after treatment was lower than before treatment ($p<0.05$). These results indicated that during the treatment, especially under the action of 5-FU, some cytotoxic T cells were activated and migrated into tumour tissues, participating in the direct killing of tumour cells. In addition, decreased levels of $CD8^+$ T cells may also be associated with cell activation-induced apoptosis, a natural phenomenon of T cells during sustained responses (Table 1 and Fig. 3).

DISCUSSION

Cervical cancer is the second most common malignancy in women worldwide after breast cancer, and the incidence is significantly higher in developing countries, accounting for approximately 15% of female cancers [17,18]. Although HPV vaccination and screening technology can effectively reduce and suppress the incidence of cervical cancer, HPV infection is still the main factor that induces cervical cancer in women. Most HPV infections are cleared by the body's autoimmune response; however, persistent high-risk HPV infection may activate the Wnt/β-catenin signalling pathway, which in turn promotes the development of cervical lesions [19]. Aberrant activation of the Wnt/β-catenin signalling pathway is closely related to the occurrence of a variety of tumours.

This study examined how the Wnt/β-catenin signalling pathway in 5-FU promotes apoptosis of high-risk HPV-positive cervical cancer cells, and the correlation of lymphocytes was discussed; furthermore, the role of T lymphocytes in this process was analysed,

Table 1
T lymphocyte levels in patients.

Cellular level/%	n	Before treatment	After treatment	*t*	*p*
$CD3^+$	78	58.24±4.58	68.94±3.58	17.060	<0.01
$CD4^+$		38.37±4.46	43.28±2.14	29.797	<0.01
$CD8^+$		21.49±2.12	24.17±2.11	8.090	<0.01
$CD4^+/CD8^+$		1.58±2.11	2.02±1.00	7.619	<0.01

Note: 5-FU treatment; t test was used for statistical analysis.

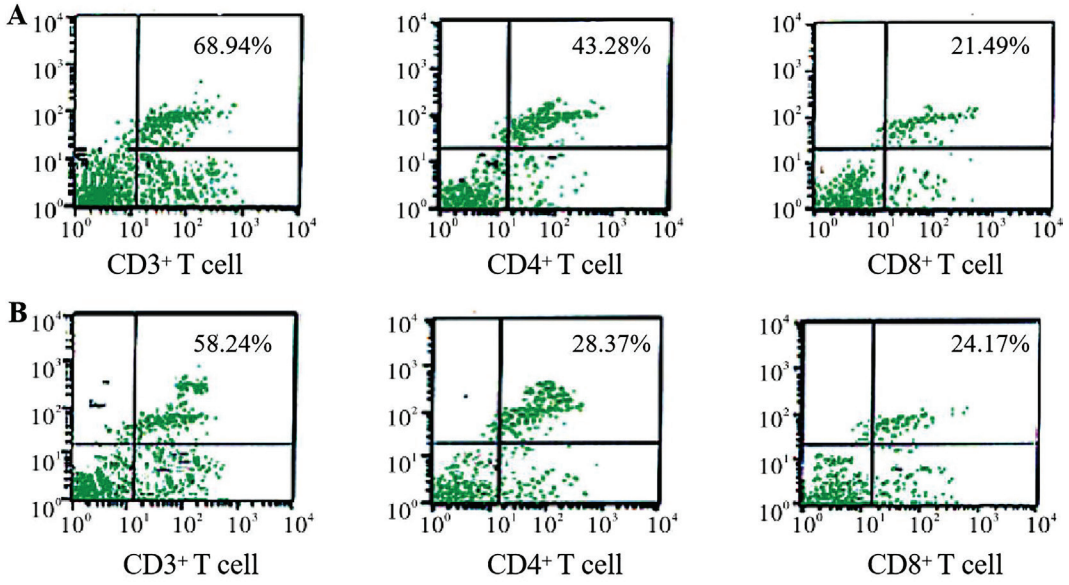

Fig. 3. Lymphocyte flow cytometry Before and after surgery. A. After surgery; B. Before surgery.

which provided a new perspective for the treatment of cervical cancer.

Broniarczyk *et al.* [20] showed that the expression level of Wnt/β-catenin protein was positively correlated with the expression level of high-risk HPV-positive cervical cancer tissues, and the high-risk HPV was positive. The content of Wnt/β-catenin protein in cervical cancer tissues was significantly higher than that in HPV-negative cervical cancer tissues [20]. The study of Li *et al.* [21] found that risperidone had a more obvious pro-apoptotic effect on bone cells, and the content of β-catenin protein was significantly reduced, which may be because risperidone has a certain inhibitory effect on β-catenin protein and slows down β-catenin. Proteins are transferred to the inner core of the cell, which upsets the balance between anti-apoptotic and pro-apoptotic protein cells [21]. The results showed that the expression level of Wnt/β-catenin protein in the paclitaxel group was higher than that in the control group after 5-FU treatment, while the expression level of Wnt/β-catenin protein in the paclitaxel group was significantly higher than that in the control group, and there was a significant difference. This indicates that the Wnt/β-catenin signalling pathway may be affected by different drugs, among which 5-FU has the most significant effect, and 5-FU can more effectively inhibit Wnt/β-catenin signalling pathways to enhance apoptosis-inducing effects on cervical cancer cells.

Shu *et al.* [22] found that after high-dose 5-FU treatment, the proliferation ability and migration ability of colorectal cancer stem cells were positively improved, while the apoptosis rate decreased significantly. These results suggest that 5-FU can induce activated stem cells by activating Wnt/β-catenin signalling cells and ultimately induce the occurrence and development of tumour diseases, which is very unfavourable to patients [22]. The study used flow cytometry to analyse the level of T lymphocytes in patients' blood and found that under the action of 5-FU, some cytotoxic T cells can be activated and migrate to tumour tissues, directly killing tumour cells, which is similar to the report of Shu et al.[22] However, it also directly shows that the decrease of T cell level has a certain correlation with cell

activation inducing apoptosis, and 5-FU can not only promote cell proliferation but also promote apoptosis. Zheng *et al*. [23] proposed that an *Artemisia annua* drug could affect and inhibit the proliferation and migration of melanoma cells by inhibiting the Wnt/β-catenin signalling pathway and ultimately induce apoptosis [23].

There are some limitations in this study; limited data were used, and the correlation between Wnt/β-catenin protein expression levels and T lymphocytes was not explored. Future studies need to expand the range of experimental data further to explore the related mechanisms of 5-FU and Wnt/β-catenin signalling in the occurrence and development of cervical cancer. Taken together, the findings support the hypothesis of a close link between the Wnt/β-catenin signalling pathway and T lymphocyte levels and reveal that 5-FU may enhance the immune response in patients with cervical cancer by modulating this signalling pathway; this offers new perspectives for the personalised treatment of high-risk HPV-positive cervical cancer.

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Ethics approval and consent to participate

This study was conducted in accordance with the declaration of Helsinki.This study was conducted with approval from the Ethics Committee of The 305 Hospital of PLA. Written informed consent was obtained from all participants.

Competing interest

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Conception and design:WC, XL; Administrative support: JD, HZ; Provision of study materials or patients: PL, LW; Collection and assembly of data: LW, JD;Data analysis and interpretation: PL, XL, HZ; Manuscript writing: All authors; Final approval of manuscript: All authors

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