1. Introduction

Bladder carcinoma (BC), a malignancy originating from the bladder, is closely related to environmental factors and smoking [1]. According to the statistics of the World Health Organization, BC ranks 16th in incidence among all malignant tumors, with an incidence of 3-4 times in males compared with females, particularly in males over 50 years of age [2]. BC was found in about 5-8 per 100,000 in 2021, showing an increasing trend year by year [3]. Clinically, BC usually presents with painless hematuria, frequent micturition, urgent urination, dysuria, etc., which can cause distant metastases as the disease progresses, ultimately endangering the patient's life [4]. At present, the overall mortality rate of BC patients is about 13.3%, and timely treatment is of great significance to ensure their life safety [5].

Clinically, surgery combined with chemoradiotherapy is the major treatment for BC, but with the increasing drug resistance of tumor cells, it is urgent to find more effective treatment schemes for the disease [6]. Epidermal growth factor receptor (EGFR) is a member of the tyrosine kinase receptor family, which has tyrosine kinase activity and can bind to epidermal growth factors (EGFs) to play a role in cell signal transduction and affect cell biological behavior [7]. Abnormally high expression of EGFR has been demonstrated in a variety of tumor diseases [8, 9]. In recent years, the application of EGFR-tyrosine kinase inhibitors (EGFR-TKIs) in various neoplastic diseases has been widely concerned, with studies indicating that its effectiveness in killing tumor cells and enhancing patient prognosis when used in chemotherapy for lung and breast cancers [10, 11]. However, few studies have reported the exact effect of EGFR-TKIs in BC, and whether they play a role in improving the outcomes of BC patients requires further exploration.

Accordingly, this study aims to confirm the future role of EGFR-TKIs in the clinical treatment of BC by analyzing their impact on the disease, so as to provide a more reliable safety guarantee for the prognosis of BC patients.
and 0.25% trypsin was used for digestion when the cell confluence reached 80-90%. Cell subcultures were carried out every 2-3 days.

2.2. Cytotoxicity test

AG1478, an EGFR-TKI purchased from Shanghai ZZBIO Co., Ltd., was dissolved in a DMSO solution to prepare into solutions with concentrations of 5, 10, 15, and 20 μmol. When T24 cells grew to the logarithmic growth phase, they were inoculated into a 96-well plate at 5×10^4/mL and added with AG1478 of different concentrations. A normal culture group without AG1478 intervention was also set up. Each group had six duplicate wells. One well was selected to add 20 μL of MTT solution at 24, 48, 72, and 96 hours, respectively. Optical density (OD) values were detected, cell growth curves were plotted, and the AG1478 concentration used for subsequent experiments was selected according to the IC50.

2.3. Grouping and intervention

After confirming the concentration of AG1478 according to the above results, the logarithmic phase T24 cells were assigned to a blank group and an intervention group for normal medium culture and incubation with medium containing AG1478, respectively.

2.4. Cell cloning assay

A single-cell suspension was prepared by digestion with 0.25% trypsin and cell counting was performed with a cell counting chamber. Cells were inoculated into a 6-well plate (500 cells/well) and cultured for 2 weeks, with the culture medium changed every 4 days, followed by cell immobilization with 4% paraformaldehyde, staining with 1% crystal violet, and final cell cloning rate calculation.

2.5. Apoptosis and cycle detection

The cells of each group were collected, digested with EDTA-free trypsin, and centrifuged. The cells re-collected were then immersed in an AnnexinV-FITC conjugate (5 μL) and a PI solution (5 μL) successively, for 15 min of room temperature culture in the dark. After the addition of 1× Annexin V binding solution (400 μL), the detection was completed within 1 h with a flow cytometer, and the apoptosis rate and cell cycle distribution of each group were calculated.

2.6. Detection of apoptosis- and pyroptosis-associated proteins

Total protein was extracted from both groups of cells, and the protein concentration was adjusted to be consistent by Bradford. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electroblotting to a PVDF membrane, and sealing for 2 hours, the protein samples were incubated overnight with rabbit anti-human Bax, Bcl-2, NLRP3, Caspase-1, GSDMD, and β-actin (1:500) at 4°C. After TBST rinsing for 40 min, the samples were placed into an HRP-labeled secondary antibody (1:500) and incubated for 1 h. Gel image processing system software was used to analyze the gray value of each band after ECL kit development.

2.7. Detection of oxidative stress response markers

Cells were collected after pancreatic digestion and centrifugation to measure superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) by enzyme-linked immunosorbent assay (ELISA).

2.8. Reactive oxygen species (ROS) assay

The cell suspension was prepared and dyed at 37°C by adding 500 μL of pre-diluted DCFH-DA fluorescent probe, and incubated for 30 min away from light. After rinsing twice with PBS, a ROS fluorescence probe was used to detect the ROS content of cells. The higher the fluorescence intensity, the greater the ROS content.

2.9. Mitochondrial membrane potential detection

The cell suspension prepared was added with cell culture solution (1 mL) and JC-1 staining solution (1 mL) and thoroughly mixed, for 25 min of incubation in a 37°C incubator. They were then collected by centrifugation at room temperature, washed twice with a JC-1 staining buffer (1×), and resuspended with 500 μL of JC-1 staining buffer (1×). The mitochondrial membrane potential of cells was detected with a JC-1 fluorescent probe. JC-1 fluorescence probe, when it aggregated in the mitochondrial matrix to form polymers, and green when it cannot form polymers but formed monomers.

2.10. Statistical methods

All assays in this study were repeated three times, with the results presented in the form of (x±s). Multi-group comparisons were performed using repeated measures ANOVA and LSD intra-group tests, and inter-group comparisons used independent sample t-tests. A significance level of P<0.05 was used in all analyses.

3. Results

3.1. Cytotoxicity test results

The MTT test results showed that the activity of T24 cells after intervention with AG1478 was significantly reduced compared to the normal cells (P<0.05), with AG1478 at a concentration of 20 μmol showing the most significant inhibitory effect on cell activity (P<0.05, Figure 1). After calculation, AG1478 had an IC50 of 14.64 μmol for T24, so 15 μmol of AG1478 was selected for the follow-up test.

3.2. Effect of AG1478 on T24 activity

In the cloning assay, the cell cloning rate was significantly lower in the intervention group than in the blank group (P<0.05, Figure 2A). Besides, the apoptosis rate in the intervention group was (20.86±1.19) %, higher compared to the intervention group (20.86±1.19) %, higher than in the blank group (P<0.05, Figure 2A). Besides, the apoptosis rate in the intervention group was significantly lower than in the normal cells (P<0.05), with AG1478 at a concentration of 20 μmol showing the most significant inhibitory effect on cell activity (P<0.05, Figure 1). After calculation, AG1478 had an IC50 of 14.64 μmol for T24, so 15 μmol of AG1478 was selected for the follow-up test.

**Fig. 1. Cytotoxicity test results.**

![Cytotoxicity test results](image)
pared with the blank group (P<0.05, Figure 2B). In the cell cycle analysis, the G0/G1 phase of cells in the intervention group was found to be significantly prolonged compared to the blank group (P<0.05, Figure 2C), confirming cell arrest in the G0/G1 phase. Moreover, Bax protein was increased in the intervention group, while Bcl-2 protein was decreased (P<0.05, Figure 2D).

3.3. Influence of AG1478 on T24 cell pyroptosis

By detecting the expression of apoptosis-associated proteins, it can be seen that NLRP3, Caspase-1, and GSDMD in the intervention group were significantly increased compared to the blank group (P<0.05, Figure 3), indicating that AG1478 can promote pyroptosis in T24.

3.4. Effect of AG1478 on oxidative stress of T24

According to cell oxidative stress test results, the intervention group had lower SOD and GSH-Px levels than the blank group, and higher MDA (P<0.05, Figure 4A-C). Similarly, ROS fluorescence intensity in the intervention group was significantly enhanced compared with the blank group (P<0.05, Figure 4D), indicating that AG1478 can also enhance the oxidative stress damage of T24.

3.5. Effect of AG1478 on mitochondrial activity

Finally, the JC-1 fluorescent staining results showed that JC-1 in the blank group was basically red, while that in the intervention group was dominantly green. The JC-1 red/green fluorescence ratio was significantly lower in the intervention group versus the control group (P<0.05, Figure 5).

4. Discussion

Recent years have witnessed the rising clinical incidence of BC, making it one of the primary diseases that threaten the health and life safety of middle-aged and elderly men [12]. Finding a more effective treatment plan is of great significance to ensure the prognosis of patients. In this study, we found that EGFR-TKIs can induce the accelerated apoptosis of BC cells, which undoubtedly provides new guidance for future clinical treatment of BC. In addition, we found through further research that the mechanism of EGFR-TKIs on BC may be related to the promotion of mitochondrial oxidative stress damage and pyroptosis in BC cells, which also provides more a comprehensive reference for clinical practice.
5. Conclusion

EGFR-TKIs can arrest BC cells in the G0/G1 phase and induce accelerated apoptosis, which is related to promoting mitochondrial oxidative stress damage and accelerating pyroptosis. In the future, EGFR-TKIs may be a new treatment scheme for BC, providing more reliable prognostic safety for BC patients.

Conflicts of Interest

The authors report no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References


9. Chong CR, Janne PA (2013) The quest to overcome resistance to medical confirmation. In addition, AG1478 is not the only EGFR-TKI in clinical research, and we should analyze the impact of other EGFR-TKIs such as HS-10296 on BC in the future. Finally, clinical trials are needed to confirm the safety of EGFR-TKIs to achieve clinical application. We will conduct supplementary research and analysis as soon as possible to address the above shortcomings.


