Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

EGFR/TKIs induce excessive apoptosis of bladder carcinoma cells by arresting cell cycle and promoting mitochondrial peroxidation damage

Xiaodong Yu, Bo Liao, Zhongbo Du, Pingyu Zhu*

Department of Urology, Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan, 637000, China

Article Info

Article history:

Received: January 24, 2024
Accepted: April 12, 2024
Published: May 31, 2024

Use your device to scan and read the article online

Abstract

In recent years, bladder carcinoma (BC) has shown an increasing incidence, with poor patient outcomes. In clinical practice, BC is still mainly treated by surgery combined with chemoradiotherapy. However, as chemotherapy resistance of tumor cells becomes more and more obvious, it is urgent to find more effective BC treatment regimes. With the increasing application and growing attention paid to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in various neoplastic diseases, EGFR-TKIs have been considered as a new treatment direction in the future. In this study, the research team used AG1478, an EGFR-TKI, to intervene with the BC cell line T24. It was found that the cell activity was statistically decreased, the apoptosis was enhanced, and the cells were dominantly arrested in the G0/G1 phase, confirming the future therapeutic potential of EGFR-TKIs in BC. Besides, the research team further observed that AG1478 also promoted pyroptosis in T24 cells, and its mechanism is related to the induction of mitochondrial oxidative stress damage. The findings lay a more reliable foundation for the future application of EGFR-TKIs in BC.

Keywords: EGFR/TKIs, Bladder cancer, Cell cycle, Mitochondrial damage

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 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.

2. Materials and methods

2.1. Cell data

Human BC T24 cells, ordered from BeNa Culture Collection, were cultured in an RPMI-1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in an incubator with the temperature maintained at 37°C, appropriate humidity, and 5%CO_2. The medium was changed every other day,

* Corresponding author.
E-mail address: zhpy197802@163.com (P. Zhu).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.5.38

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 5×10^7/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, 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 fluoresced red 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 ($\bar{\chi}\pm s$). 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 com-

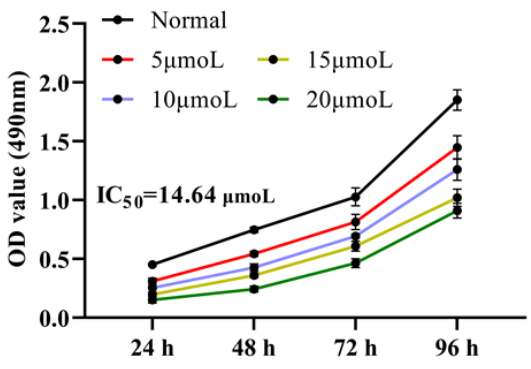

Fig. 1. Cytotoxicity test results.

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.

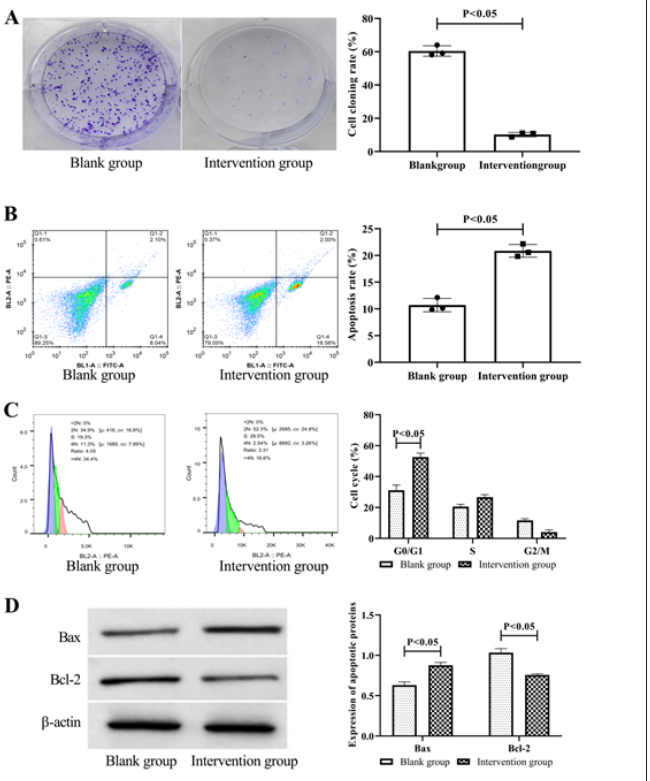

Fig. 2. Effect of AG1478 on T24 activity. (A) Effect of AG1478 on T24 cloning ability. (B) Effect of AG1478 on T24 apoptosis rate. (C) Effect of AG1478 on T24 cell cycle. (D) Effect of AG1478 on apoptotic proteins in T24.

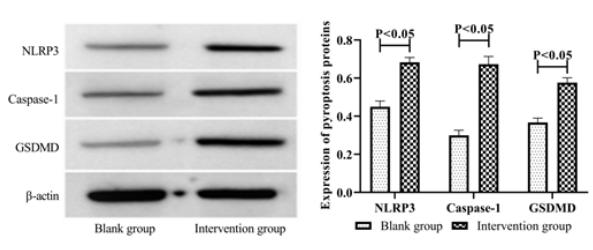

Fig. 3. Influence of AG1478 on T24 cell pyroptosis.

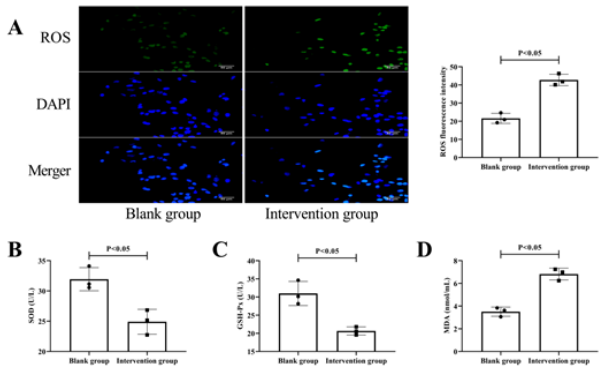

Fig. 4. Effect of AG1478 on oxidative stress of T24. (A) Effect of AG1478 on ROS in T24. (B) Comparison of SOD. (C) Comparison of GSH-Px. (D) Comparison of MDA.

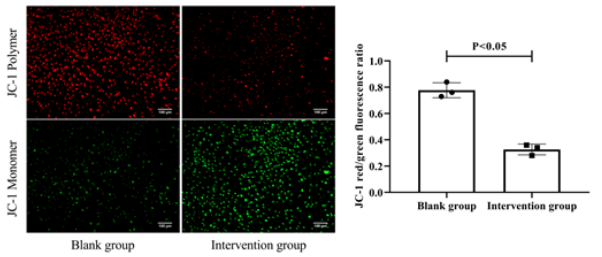

Fig. 5. Effect of AG1478 on mitochondrial activity.

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.

At present, the inhibition of BC cell growth and invasion is achieved by reducing DNA replication and synthesis in tumor nuclei or inhibiting DNA mitosis, which causes toxic damage to tumor cells; however, the disadvantage is that the destruction of non-tumor cells cannot be ruled out, impairing the body's immune function [13]. The study of tumor molecular biology has ushered in a new era in the treatment of a variety of malignancies, and targeted therapy methods are of great significance in the treatment of tumor diseases [14]. The abnormal expression of the EGFR receptor family in BC has been identified,

so therapies targeting EGFR receptors and inhibiting the activity of EGFR domains in tumor cells may be a new therapeutic direction for BC [15]. AG1478 is an EGFR-TKI commonly used in clinical research, which has been confirmed in the previous literature to inhibit the growth of cervical cancer, breast cancer, etc. [16, 17]. In this study, AG1478 intervention resulted in a significant decrease in BC cell activity, an increase in apoptosis, and a great number of cells being arrested in the G0/G1 phase, confirming the ability of AG1478 to accelerate the apoptosis of BC cells. Studies have shown that EGFR is expressed in BC, which can bind to the corresponding ligands to initiate the growth cycle of tumor cells, activate the signal transduction system of tumor cells, and transmit growth signals to the nucleus, thus stimulating the growth and invasion of BC cells [18]. Therefore, under the intervention of EGFR-TKIs, the EGFR-binding ligands in BC can be blocked, which reduces autophosphorylation and dimer formation, thereby blocking the signal transmission to the nucleus and inhibiting the growth and activity of cells [19]. Meanwhile, EGFR-TKIs can affect the transcription and protein expression of target genes, and reduce the invasion, metastasis, and angiogenesis of BC cells by reducing the EGFR adenosine triphosphate (ATP)-binding site [20].

On the other hand, we also observed markedly elevated expression of apoptosis-associated proteins in the intervention group, which also showed that EGFR-TKIs can induce biological changes in BC cells by promoting cell pyroptosis. In previous studies, EGFR-TKIs have also been widely concerned about the promotion of pyroptosis in tumor cells, and the mechanism is related to their regulation of mitochondrial damage [21]. Mitochondrial damage is known to be independent of the Bcl-2 family and dependent on the binding of GSDMD-NT to cardiolipin [22]. The typical and atypical activation of inflammasome from mitochondrial damage, cell pyroptosis, and inflammatory cytokine release can be inhibited by knocking out the genes of cardiolipin synthase 1 or recombinant phospholipid scramblase 3 (PLSCR3) that produce and transfer cardiolipin to the outer mitochondrial membrane, and PLSCR3 deficiency in tumors compromises pyroptosis-triggered anti-tumor immunity [23]. Thus, mitochondrial damage plays a key role in pyroptosis. Through detection, we found that in the intervention group, JC-1 showed green fluorescence, and the red/green fluorescence ratio was significantly reduced compared to the control group, indicating that a large amount of healthy mitochondria were degraded into monomeric mitochondria [24]. Moreover, the contents of antioxidant SOD and GSH-Px in the intervention group decreased statistically, while the contents of oxide MDA and ROS increased markedly, indicating obvious oxidative stress damage. Based on the above results, we can conclude that the extensive release of ROS in EGFR-TKI-activated cells causes oxidative damage to the mitochondrial membrane, leading to an increase in cell apoptosis and a decrease in intracellular mitochondrial membrane potential, initiating the apoptotic process of the cellular mitochondrial pathway. This may be the key to the accelerated apoptosis of BC cells induced by EGFR-TKIs.

However, due to limited experimental conditions, it is not yet possible to evaluate the impact of EGFR-TKIs on the invasion and migration ability of BC cells, and whether the abnormal expression of certain signaling pathways is involved is not clear, requiring further experimental 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.

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.

Funding

This study was supported by the 2022 Research Topics for Primary Health Centres in Sichuan Province(NO. SWFZ22-Y-29).

References

1. Xu N, Yao Z, Shang G, Ye D, Wang H, Zhang H, Qu Y, Xu F, Wang Y, Qin Z, Zhu J, Zhang F, Feng J, Tian S, Liu Y, Zhao J, Hou J, Guo J, Hou Y, Ding C (2022) Integrated proteogenomic characterization of urothelial carcinoma of the bladder. J Hematol Oncol 15 (1): 76. doi: 10.1186/s13045-022-01291-7
2. Goodstein T, Wang SJ, Lee CT (2021) Bladder preservation in urothelial carcinoma: current trends and future directions. Curr Opin Support Palliat Care 15 (4): 253-259. doi: 10.1097/SPC.0000000000000579
3. Schwarzova L, Varchulova Novakova Z, Danisovic L, Ziaran S (2023) Molecular classification of urothelial bladder carcinoma. Mol Biol Rep 50 (9): 7867-7877. doi: 10.1007/s11033-023-08689-7
4. Yegin Z, Aydin O, Koc H, Buyukalpelli R (2018) Expression profiles of proto-oncogene TWIST1 and tumor metastasis suppressor gene LASS2 in bladder cancer. Cell Mol Biol (Noisy-le-grand) 64 (11): 66-73. doi:
5. ElSharnoby O, Fraser N, Williams A, Scriven S, Shenoy M (2022) Bladder urothelial cell carcinoma as a rare cause of haematuria in children: Our experience and review of current literature. J Pediatr Surg 57 (7): 1409-1413. doi: 10.1016/j.jpedsurg.2021.09.007
6. Puzyrenko A, Nomani L, Iczkowski K, Giorgadze T (2022) Small cell carcinoma of the urinary bladder: Distinctive cytological characteristics and Cyto-histologic correlation. Ann Diagn Pathol 59: 151947. doi: 10.1016/j.anndiagpath.2022.151947
7. Sabbah DA, Hajjo R, Sweidan K (2020) Review on Epidermal Growth Factor Receptor (EGFR) Structure, Signaling Pathways, Interactions, and Recent Updates of EGFR Inhibitors. Curr Top Med Chem 20 (10): 815-834. doi: 10.2174/1568026620666200303123102
8. Singh D, Attri BK, Gill RK, Bariwal J (2016) Review on EGFR Inhibitors: Critical Updates. Mini Rev Med Chem 16 (14): 1134-1166. doi: 10.2174/1389557516666160321114917
9. Chong CR, Janne PA (2013) The quest to overcome resistance to

EGFR-targeted therapies in cancer. Nat Med 19 (11): 1389-1400. doi: 10.1038/nm.3388

10. Abourehab MAS, Alqahtani AM, Youssif BGM, Gouda AM (2021) Globally Approved EGFR Inhibitors: Insights into Their Syntheses, Target Kinases, Biological Activities, Receptor Interactions, and Metabolism. Molecules 26 (21). doi: 10.3390/molecules26216677
11. Zanetti-Domingues LC, Bonner SE, Iyer RS, Martin-Fernandez ML, Huber V (2020) Cooperation and Interplay between EGFR Signalling and Extracellular Vesicle Biogenesis in Cancer. Cells 9 (12). doi: 10.3390/cells9122639
12. Lopez-Beltran A, Blanca A, Cimadamore A, Montironi R, Luque RJ, Volavsek M, Cheng L (2022) T1 bladder carcinoma with variant histology: pathological features and clinical significance. Virchows Arch 480 (5): 989-998. doi: 10.1007/s00428-021-03264-6
13. Laukhtina E, Pradere B, Lemberger U, Karakiewicz PI, Fajkovic H, Shariat SF (2022) Molecular biomarkers to help select neoadjuvant systemic therapy for urothelial carcinoma of the bladder. Curr Opin Urol 32 (5): 561-566. doi: 10.1097/MOU.0000000000001013
14. Tao TT, Chen J, Hu Q, Huang XJ, Fu J, Lv BD, Duan Y (2020) Urothelial carcinoma of the bladder with abundant myxoid stroma: A case report and literature review. Medicine (Baltimore) 99 (28): e21204. doi: 10.1097/MD.0000000000021204
15. van der Fels CAM, Leliveld A, Buikema H, van den Heuvel MC, de Jong IJ (2022) VEGF, EGFR and PSMA as possible imaging targets of lymph node metastases of urothelial carcinoma of the bladder. BMC Urol 22 (1): 213. doi: 10.1186/s12894-022-01157-7
16. Shi K, Wang G, Pei J, Zhang J, Wang J, Ouyang L, Wang Y, Li W (2022) Emerging strategies to overcome resistance to third-generation EGFR inhibitors. J Hematol Oncol 15 (1): 94. doi: 10.1186/s13045-022-01311-6
17. Marin AP, Arranz EE, Sanchez AR, Aunon PZ, Baron MG (2010) Role of anti-Her-2 therapy in bladder carcinoma. J Cancer Res Clin Oncol 136 (12): 1915-1920. doi: 10.1007/s00432-010-0850-7
18. Li W, Wang Y, Tan S, Rao Q, Zhu T, Huang G, Li Z, Liu G (2018) Overexpression of Epidermal Growth Factor Receptor (EGFR) and HER-2 in Bladder Carcinoma and Its Association with Patients' Clinical Features. Med Sci Monit 24: 7178-7185. doi: 10.12659/MSM.911640
19. Hashmi AA, Hussain ZF, Irfan M, Khan EY, Faridi N, Naqvi H, Khan A, Edhi MM (2018) Prognostic significance of epidermal growth factor receptor (EGFR) over expression in urothelial carcinoma of urinary bladder. BMC Urol 18 (1): 59. doi: 10.1186/s12894-018-0373-0
20. Mooso BA, Vinall RL, Mudryj M, Yap SA, deVere White RW, Ghosh PM (2015) The role of EGFR family inhibitors in muscle invasive bladder cancer: a review of clinical data and molecular evidence. J Urol 193 (1): 19-29. doi: 10.1016/j.juro.2014.07.121
21. Rose M, Maurer A, Wirtz J, Bleilevens A, Waldmann T, Wenz M, Eyll M, Geelvink M, Gereitzig M, Ruchel N, Denecke B, Eltze E, Herrmann E, Toma M, Horst D, Grimm T, Denzinger S, Ecke T, Vogeli TA, Knuechel R, Maurer J, Gaisa NT (2020) EGFR activity addiction facilitates anti-ERBB based combination treatment of squamous bladder cancer. Oncogene 39 (44): 6856-6870. doi: 10.1038/s41388-020-01465-y
22. Chong SJF, Iskandar K, Lai JXH, Qu J, Raman D, Valentin R, Herbaux C, Collins M, Low ICC, Loh T, Davids M, Pervaiz S (2020) Serine-70 phosphorylated Bcl-2 prevents oxidative stress-induced DNA damage by modulating the mitochondrial redox metabolism. Nucleic Acids Res 48 (22): 12727-12745. doi: 10.1093/nar/gkaa1110
23. Tigano M, Vargas DC, Tremblay-Belzile S, Fu Y, Sfeir A (2021) Nuclear sensing of breaks in mitochondrial DNA enhances immune surveillance. Nature 591 (7850): 477-481. doi: 10.1038/s41586-021-03269-w
24. Zhang Y, Xia M, Zhou Z, Hu X, Wang J, Zhang M, Li Y, Sun L, Chen F, Yu H (2021) p53 Promoted Ferroptosis in Ovarian Cancer Cells Treated with Human Serum Incubated-Superparamagnetic Iron Oxides. Int J Nanomedicine 16: 283-296. doi: 10.2147/IJN.S282489