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Inhibition of Bruton's Tyrosine Kinase Suppresses Cancer Stemness and Promotes Carboplatin-induced Cytotoxicity Against Bladder Cancer Cells

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Abstract. *Background/aim: Bruton's tyrosine kinase (BTK) has been discovered to serve a critical role in the survival and infiltration of B-cell lymphoma. Recently, it was reported that BTK inhibitors exerted potential beneficial effects against numerous types of solid tumor, including glioblastoma multiforme and breast cancer; however, whether BTK is crucial for the progression of bladder cancer (BLCA) remains unclear. The present study investigated the in vitro function of BTK in stemness properties of BLCA cells. Furthermore, the therapeutic effects of a standard chemotherapeutic drug, carboplatin in combination with the BTK inhibitor, ibrutinib were also investigated. Materials and Methods: The association between BTK and BLCA progression was evaluated using free databases. The in vitro stemness and metastatic properties of BLCA cells were also investigated. Finally, the cytotoxicity of carboplatin in combination with ibrutinib was determined. Results: The meta-survival analysis of the association between BTK and BLCA progression revealed that the expression levels of BTK were associated with a higher risk of BLCA progression. The $CD133^+$-side population of BLCA cells formed spheroids when cultured in serum-free conditioned medium. In addition, expression levels of BTK and activated mTOR signaling in side population cells was up-regulated compared with the parental BLCA cells. Furthermore, the transfection of short hairpin RNA targeting BTK into BLCA cells markedly reduced cell migratory ability. More importantly, in advanced BLCA cells, which were more resistant to carboplatin, it was discovered that the cell viability was significantly reduced in the presence of ibrutinib ($p<0.05$). Conclusion: The findings of the present study suggested that BTK may have a critical role in the progression of BLCA; however, the underlying mechanisms and potential therapeutic strategies involved require further investigations.*

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Key Words: Bruton's tyrosine kinase, bladder cancer, chemotherapy, drug resistance, cytotoxicity.

Bruton's tyrosine kinase (BTK) is a cytoplasmic non-receptor tyrosine kinase member that is highly expressed in lymphocytes and other subtypes of cancer cells (1). BTK has been discovered to serve a critical role in the proliferation and survival of leukemia cells in a large number of B-cell lymphomas. BTK is downstream of the signaling transduction pathway of the B-cell antigen receptor. BTK inhibitors, such as ibrutinib, have been approved for the first-line treatment of patients with relapsed/refractory chronic lymphocytic leukemia and mantle-cell lymphoma since

2016, due to their high efficacy and tolerance (2). Recently, it was reported that the inhibition of BTK in pancreatic, breast and colon cancer dramatically improved survival when combined with chemo- or immunotherapy (3, 4).

Urothelial carcinoma of the bladder is one of the most fatal sex-specific types of malignancy in the world (5). Due to the limited number of treatments currently available, patients with bladder cancer (BCLA) who undergo radical cystectomy have a higher overall survival rate compared with those treated with chemoradiation (6). Maintaining a good quality of life without negatively affecting the chances of a cure or long-term survival is imperative for patients with muscle-invasive BCLA. Although the gemcitabine/cisplatin regimen has demonstrated clinical benefits without severe adverse responses, carboplatin is an alternative therapeutic used when regarding the eligibility of cisplatin for the treatment of BLCA (7).

To the best of our knowledge, no previous study has investigated the role of BTK in combination with standard chemotherapy in BLCA. In the present study, the clinical association between BTK and the progression of BLCA was determined, and the role of BTK in the stemness and migration of BLCA cells was further investigated. Furthermore, the cytotoxicity of carboplatin in combination with the BTK inhibitor ibrutinib against carboplatin-resistant human BLCA cells was also determined.

Materials and Methods

Bioinformatics analysis of BTK expression. In the beginning, we evaluated the BTK expression pattern in human bladder cancer and normal tissues using the Gene Expression database of Normal and Tumor tissues (GENT2). The data were analyzed from the microarray data of published urothelial carcinoma transcriptome GSE32894, GSE31684 and GSE13507. The hazard ratio of overall survival was investigated.

Cell lines, reagents and chemicals. T24 human BLCA and HT 1197 human BLCA cell lines were purchased from Bioresources Collection and Research Center (Hsin Chu, Taiwan, ROC). The BTK inhibitor ibrutinib was purchased from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). Carboplatin was purchased from Accela ChemBio. Inc. (San Diego, CA, USA). alamarBlue™ Cell Viability reagent, L-glutamate, fetal bovine serum, TrypLE Express Enzyme solution, McCoy's 5a basal medium (for the culture of T24 cells) and minimum essential medium (Eagle) with Earle's balanced salt solution (for the culture of HT 1197 cells) were purchased from Gibco, Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anti-IgG1-allophycocyanin (APC) and anti-CD133-APC human monoclonal antibodies were obtained from BD Biosciences (San Jose, CA, USA).

Human BLCA cell culture. The T24 and HT 1197 human BLCA cell lines were cultured in complete media (90% basal medium containing 1.5 g/l sodium bicarbonate and 10% fetal bovine serum) in a humidified cell culture incubator at 37˚C and 5% CO2. The cells were detached using TrypLE Express Enzyme solution for cell passage.

Cell surface marker recognition for analyzing the stemness of BLCA cells. The parental BLCA cells were harvested and incubated with anti-IgG1-APC and anti-CD133-APC human monoclonal antibodies. Following 30 min of incubation at room temperature in the dark, the cells were centrifuged at 300 × *g* for 10 min at 25˚C. The supernatant was then aspirated and the cell pellet was resuspended and gently mixed with 1 ml phosphate-buffered saline. Finally, the cells were analyzed and sorted using a flow cytometer (FACSAria™ II cell sorter; BD Biosciences, San Jose, CA, USA). The harvested $CD133^+$-side population (SP) cells were maintained in serum-free medium with basal medium-containing basic fibroblast growth factor (10 ng/ml), leukemia inhibitory factor (10 ng/ml) and epidermal growth factor (20 ng/ml), and incubated in a low-adherent Petri dish in a humidified cell culture incubator at 37˚C and 5% CO_2 for 7 days and the medium refreshed every 3 days.

Western blotting. SP and parental BLCA cells were harvested and lysed in cold RIPA lysis buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich; Merck KGaA). Total protein was quantified using a Bradford protein assay and the protein lysate was separated via gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were subsequently electrotransferred onto a polyvinylidene fluoride membrane (Sigma-Aldrich) and blocked. The membranes were then incubated with primary antibodies to the following: BTK, phosphorylated mammalian target of rapamycin (mTOR), total mTOR and β-actin, respectively. Following incubation with primary antibody, the membranes were incubated with specific horseradish peroxidase-conjugated secondary antibody. All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Protein bands were quantified using chemiluminescence (EMD Millipore, Billerica, MA, USA) and a ChemiDoc™ Imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal protein loading was confirmed by analyzing the expression levels of β-actin.

Knockdown of BTK expression. BTK expression was knocked down in BLCA cells using short hairpin RNA against BTK (shBTK). A pLKO.1-puromycin-based vector expressing shBTK was purchased from Academia Sinica (Taipei, Taiwan, ROC). The BLCA cells were transfected with 10 μg/ml shBTK for 4 h prior to the replacement of the cell culture medium.

Wound-healing assay. A wound-healing assay was performed to determine the cell migratory ability of transfected and non-transfected cells. Briefly, a 200-μl pipette tip was used to scratch the surface of the cell monolayer. Following 24-hour incubation, the cell migratory ability was quantified using an inverted microscope equipped with a CCD camera (Olympus Corporation, Tokyo, Japan) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cytotoxicity assay of the BTK inhibitor, ibrutinib, in combination with carboplatin in BLCA cells. BLCA cells were treated independently with carboplatin (0-100 μM) or ibrutinib (0-20 μM), or in combination, for 24 and 48 h, and the cell viability was subsequently determined. Cell viability assay was performed using alamarBlue reagent. Briefly, after washing the cells with phosphate-buffered saline, the cell culture medium was replaced with basal medium-containing alamarBlue reagent and incubated in a cell culture incubator for another 3 hours. The fluorescence intensity was measured using an

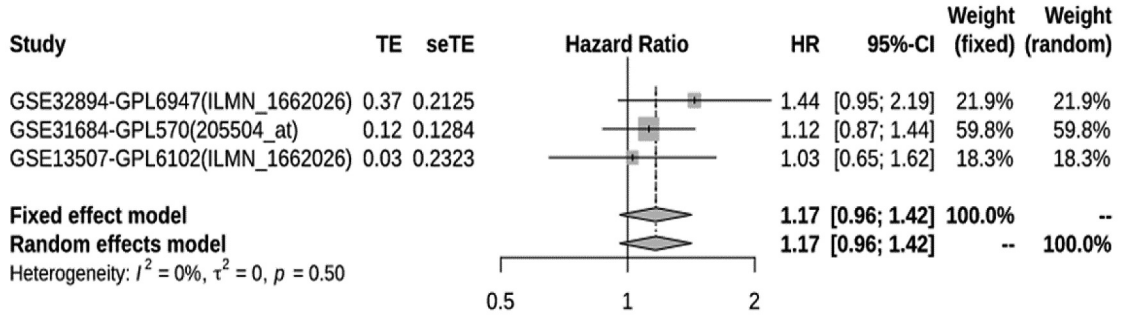

Figure 1. *Forest plot of studies included in the meta-analysis of the expression of Bruton's tyrosine kinase (BTK) and the risk of bladder cancer mortality. Data were adapted from GENT2 platform (http://gent2.appex.kr/gent2/). CI: Confidence interval; HR: hazard ratio; TE: Ln(HR); seTE: standard error of HR.*

excitation wavelength of 544 nm and an emission wavelength of 590 nm on an ELISA reader (SpectraMax M2e; Molecular Devices, LLC, Sunnyvale, CA, USA). The cell viability (%) was calculated using the following equation: [(Fluorescence$_{treated}$–Fluorescence$_{control}$)/ Fluorescencecontrol] ×100.

Statistical analysis. All data were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and are presented as the mean±SEM of three or more independent experiments. Statistical differences between two groups were analyzed using a one-way ANOVA, followed by a *post hoc* test. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Results

BTK expression level predicts a higher risk of BLCA. The association between the predictive value of BTK expression level and overall survival of patients with BLCA was determined from three studies and the fixed hazard ratio derived was 1.166 (95% confidence interval=0.960-1.417; Figure 1). Furthermore, T24 (grade III) and HT1197 (grade IV) BLCA cells were used to investigate the stemness characteristics of BLCA cells. As shown in Figure 2, the CD133$^+$ SP was harvested using a flow cytometer (Figure 2A) and the cells were cultured in serum-free medium with growth factors. The morphology of parental and SP cells is presented in Figure 2B. Compared with parental cells, the floating SP cells were found to be spheroid in morphology following 1 week of incubation.

CD133$^+$ SP cells have upregulation of BTK and activated mTOR signaling. The protein expression levels of BTK and mTOR in parental and SP cells were further investigated. Compared with the parental cells, the data revealed that SP cells had a higher BTK content and activated mTOR signaling in both BLCA cell lines (Figure 3).

Inhibition of BTK reduces the migratory ability of BLCA cells. The role of BTK in the migratory ability of BLCA cells was subsequently investigated. T24 and HT 1197 cells were transfected with shBTK and the wound-healing ability was determined. The results revealed that the knockdown of BTK reduced the cell-migratory ability of BLCA cells (Figure 4).

BTK inhibitor enhances the carboplatin-induced cytotoxicity against BLCA cells. The present study further investigated the effect of the BTK inhibitor, ibrutinib, on the cytotoxicity of carboplatin on BLCA cells. First, T24 and HT 1197 cells were treated with 0-100 μM carboplatin for 24 and 48 h. As shown in Figure 5, the HT1197 cells were discovered to be more resistant to carboplatin; the half-maximal inhibitory concentration for T24 cells of 30 μM compared with 80 μM for HT1197 cells following a 48-hour incubation. Subsequently, whether carboplatin treatment in combination with ibrutinib enhanced the cytotoxicity was investigated. Compared with the group treated with carboplatin alone, the combination of 20 μM ibrutinib with carboplatin significantly increased the cytotoxicity of carboplatin-resistant HT1197 cells ($p<0.05$).

Discussion

BLCA comprises a group of heterogeneous tumors, with over more than 40 histological subgroups. Genetic instability and exposure to carcinogens are considered to be the two major risk factors for BLCA progression. Muscle-invasive BCLA accounts for ~25% of newly diagnosed BLCAs (6). The recommended standard treatment for non-metastatic muscle-invasive BCLA is transurethral resection of the tumor, followed by radical cystectomy with neoadjuvant platinum-based chemotherapy, if acceptable. Recent advances in molecular medicine have resulted in the identification of novel predictive biomarkers for the treatment of BLCA.

Recently, it was suggested that BTK may be a crucial target in the progression of numerous types of solid tumor (8).

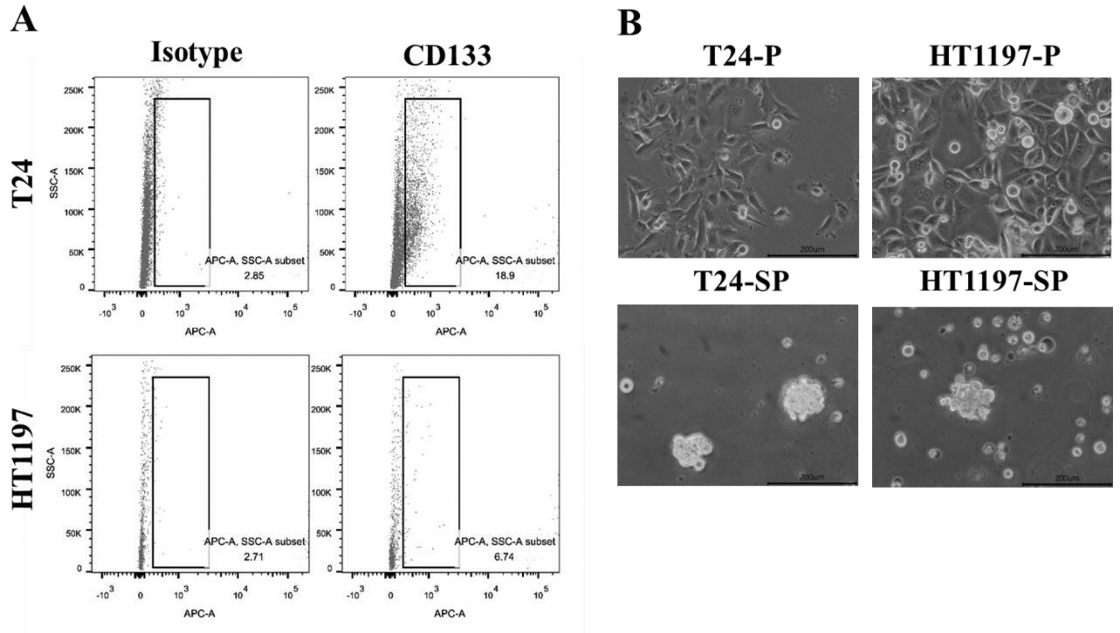

Figure 2. *CD133+ side population of bladder cancer cells. A: T24 and HT1197 bladder cancer cells were stained with IgG-isotype or CD133 antibody, respectively. Flow cytometric analysis of the proportion of CD133+ cells followed by harvesting of the CD133+ side population, respectively. B: Morphology of parental (P) and side population (SP) cells cultured in complete and serum-free medium, respectively, and cultured for 1 week (magnification, ×100; scale bar, 200 μm).*

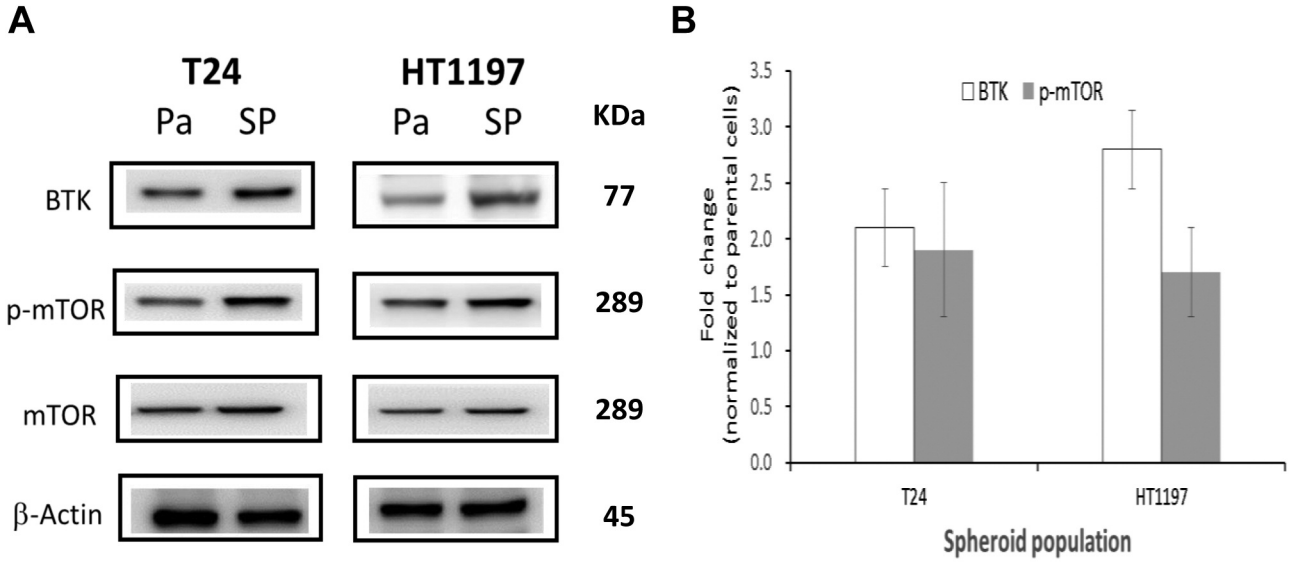

Figure 3. *Expression of Bruton's tyrosine kinase (BTK) and mammalian target of rapamycin (mTOR) in bladder cancer cells. A: The protein levels of BTK and mTOR in parental (P) and CD133+ side population (SP) T24 and HT1197 cells, respectively. B: Data were averaged from three independent experiments.*

Originally, BTK was classified as a tyrosine protein TEC-kinase family member that is expressed in lymphocytes and other cell subtypes. BTK and four additional molecules, namely bone marrow-expressed kinase, redundant resting lymphocyte kinase and IL2-inducible T-cell kinase, comprise the non-receptor kinases. The TEC kinase family has been discovered to serve a predominant role in the intracellular signaling of various membrane receptors, in addition to

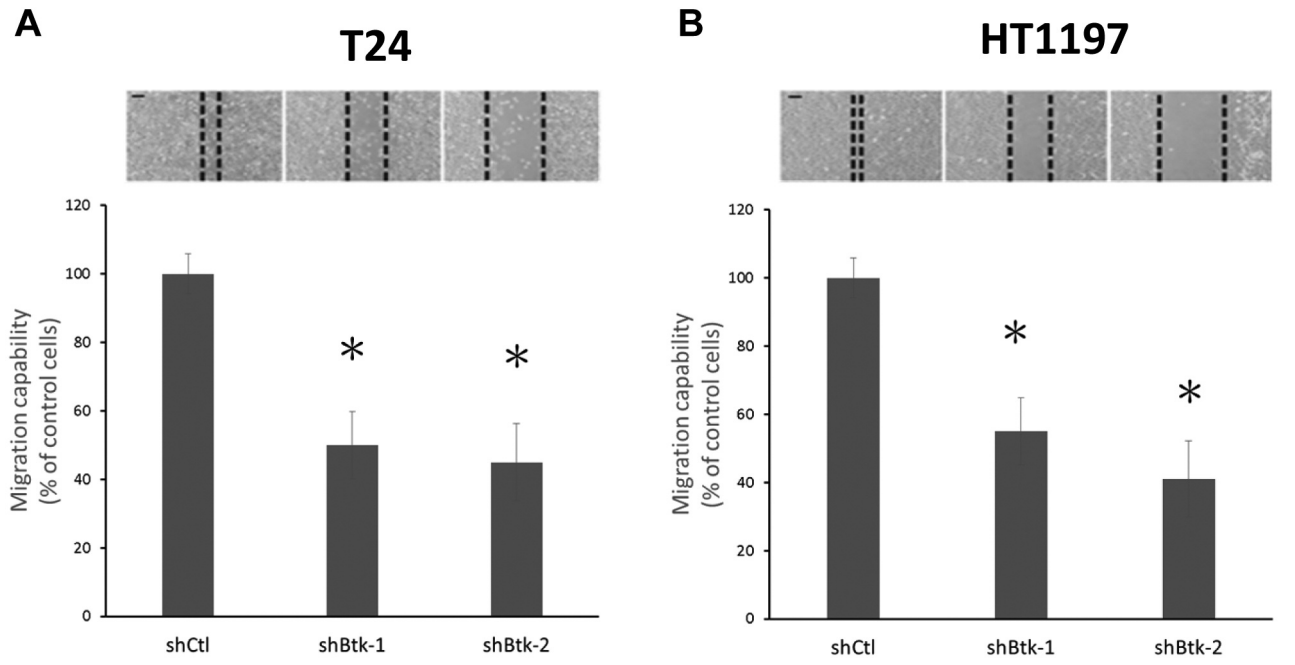

Figure 4. *Inhibitory effect of knockdown of Bruton's tyrosine kinase (BTK) on wound healing of bladder cancer cells. T24 (A) and HT1197 (B) cells were pre-treated with small hairpin RNA against BTK (shBtk) and then a pipette tip was used to make a scratch in the cell layer to investigate the cell migration capability, (magnification, ×100; scale bar, 50 μm). Significantly different at *p<0.05 between short hairpin RNA control (shCtl) group and shBtk group foreach cell line.*

regulating biological processes and tumor progression. BTK sequentially catalyzes the phosphorylation and activation of phospholipase Cγ2, which results in the downstream activation of the guanosine-5'-triphosphate-binding protein and nuclear factor κB signaling pathways (9). Ibrutinib, an effective inhibitor against BTK and IL2-inducible T-cell kinase, was the first US Food and Drug Administration-approved drug for the treatment of B-cell lymphoma. Ibrutinib was reported to enhance the therapeutic effects of the immune checkpoint inhibitor, programmed death ligand-1 antibody, against breast and colon cancer (10). In addition, its proven ability to inhibit the erythroblastic leukemia viral oncogene homolog receptor family, and epidermal growth factor receptor-2 as well as epidermal growth factor receptor activation, also suggests broader applications of ibrutinib in oncology (11). BTK may represent a novel therapeutic target for glioma and ibrutinib may serve as an adjuvant is therapy for malignant glioblastoma multiforme due to the demonstrated stemness and tumorigenic functions of BTK (12).

Currently, there are relatively few studies reporting the function of BTK in the stemness, metastasis and drug resistance of BLCA. The findings of the present study revealed that the expression of BTK protein was up-regulated in $CD133^{+}$-SP BLCA cells. Importantly, the knockdown of BTK dramatically reduced the cell-migratory ability. In addition, the cytotoxicity of carboplatin against chemoresistant BLCA cells was significantly enhanced in the presence of the BTK inhibitor. These data support BTK having a crucial role of in the stemness and drug resistance of BLCA.

In conclusion, the present study suggested that BTK may play a crucial role in the stemness and migration of BCLA cells. The clinical evaluation revealed that up-regulation of BTK level was associated with a higher risk of BCLA progression. Most notably, BTK in combination with carboplatin improved its cytotoxicity against carboplatin-resistant cells. Therefore, the underlying mechanisms should be investigated further through pre-clinical evaluations to develop alternative strategies for the treatment of BLCA.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in regard to this study.

Authors' Contributions

SCC, DYC and PHS made contributions to the conception and design of the study and prepared the article. YP, YHC and PHS performed the experiments and data analysis. LML, YCW and JWP reviewed the literature and interpreted the results. CHH and PHS revised the article. All Authors read and approved the final article.

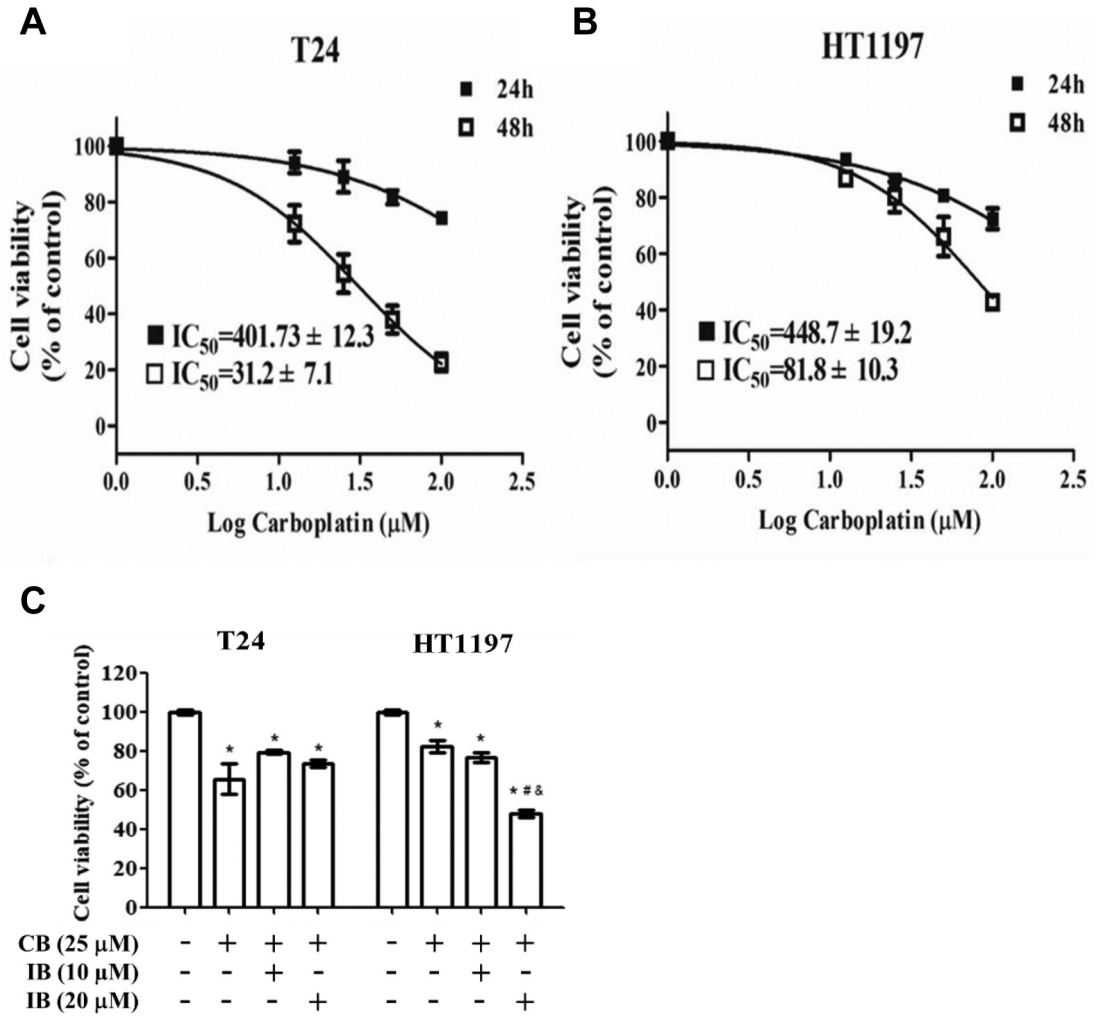

Figure 5. *Bruton's tyrosine kinase (BTK) inhibitor promoted cytotoxicity of carboplatin in carboplatin-resistant bladder cancer cells. T24 (A) and HT1197 (B) cells were treated with different concentrations of carboplatin (CB, 0-100 μM) for 24 and 48 h, respectively, and then the cell viability was evaluated. C: T24 and HT1197 were treated with carboplatin (25 μM) in the presence of ibrutinib (IB, 10 or 20 μM) for 48 h, respectively. Significantly different at p<0.05 compared with the: *control group, #group treated with carboplatin alone, and &group treated with ibrutinib (10 μM).*

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