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Original article

A study of an effective sunitinib–chemotherapeutic combination regimen for bladder cancer treatment using a mouse model

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ABSTRACT

Objective: To determine if tyrosine kinase receptor inhibitor, sunitinib malate, combined with chemotherapeutic drugs may present synergistic enhancement of cytotoxicity to transitional cell carcinoma cells (TCC).

Methods: The mRNA and protein contents of vascular endothelial growth factor-α (VEGFα) in various TCC cell lines were detected individually by quantitative-polymerase chain reaction and Western blot. The inhibitory concentrations of various chemotherapeutic drugs, including gemcitabine, doxorubicin, and cisplatin, and their combination with sunitinib to TCC cancer cells were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The synergist efficacy was measured using the observed/expected ratio calculation method. Finally, the synergistic effect of sunitinib and selected anticancer drug gemcitabine was elucidated in C3H/MBT-2 animal tumor model with monitoring tumor growth volume and survival rate.

Results: The mRNA of VEGFα had high expression in high-grade TCC cell lines (T24, TCC 8701, and TCC 8702) when compared with low-grade TCC cell lines (TCC 8301 and TSGH 9201). The expression of VEGFα protein level was closely correlated with the mRNA content in each individual cell line. Sunitinib, combined with gemcitabine, has shown the highest synergistic cytotoxic effect to TCC cells in an MTT assay. In the xenografted tumor model, MBT-2 bearing mice treated by sunitinib and gemcitabine combination had the lower mean tumor volume (265 ± 95 mm^3 vs. 2605 ± 320 mm^3) and higher survival rate (100% vs. 56%) at 30 days follow-up when compared with control mice.

Conclusion: Combination of the tyrosine kinase receptor inhibitor sunitinib with gemcitabine chemotherapy synergistically enhances tumor cytotoxicity and may provide a new treatment modality for advanced bladder cancer.

1. Introduction

In Taiwan, the incidence of urothelial carcinomas is increasing yearly. A total of 735 victims died of bladder cancer in 2010 with a standard mortality rate of 2.2/100,000 according to the statistics from the Department of Health, Executive Yuan, Taiwan, R.O.C.[1] In patients with urothelial carcinoma, around 90% have transitional cell carcinoma (TCC). Of these, 15–20% will invade into the outer tissues of bladder or the pelvic lymph nodes with distant metastasis.[2] Therefore, high recurrence with disease progression is a clinical characteristic of bladder cancer. Currently, the available treatment for advanced and metastatic bladder cancer is systemic chemotherapy. Although randomized trials have established an incremental benefit from the addition of chemotherapy in this setting, many patients still have disease relapse, and therefore it is necessary to investigate more efficient treatment modalities for this disease entity.

Large efforts have been devoted to the development of new chemotherapeutic reagents and the design of new strategies for prevention recurrence and metastatic bladder cancers. Primary bladder tumors and metastatic sites appear to be highly vascular. The critical role of angiogenesis in tumor growth and metastasis has prompted many efforts to develop antiangiogenic therapies, thus providing a rational drug target for using angiogenic inhibitors in bladder cancer tumors.[3] Sunitinib malate is one of several new antiangiogenic agents undergoing tests of efficacy in the treatment of various types of cancer, including gastrointestinal stromal tumors and renal cell carcinoma.[4] This study attempts to verify the possible synergistic effect of anticancer drugs and sunitinib in treating TCC.

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2. Materials and methods

Human TCC cell lines (high-grade: T24, TCC8701 and TCC8702; low-grade: TSGH8301 and TSGH9201) and a mouse bladder cancer cell line (MBT-2; JCRB, Tokyo, Japan), were cultivated in an Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (Copenhagen, DK), with 100 U/mL penicillin, 50 mg/mL streptomycin, nonessential amino acid, and 0.01M 4-(2-hydroxyethyl)-1- piperazine ethanesulfonic acid (HyClone, DK). Confluent cells were detached with 0.01M EDTA.

Sunitinib malate (Sutent) was generously provided by Pfizer Inc. (City, Taiwan). Twenty-five mg of sunitinib was dissolved in 5 mL of phosphate buffered saline (PBS) (pH 6.5) to make 5 mg/mL stock. Gemcitabine and doxorubicin were generously provided by TTY Biopharm (City, Taiwan). Cisplatin was purchased from Sigma-Aldrich (St Louis, MO, USA).

Quantitative polymerase chain reaction (qPCR) detection of vascular endothelial growth factor-α (VEGFα) mRNA was conducted by a three-step procedure using the ABI 7500 instrument (ABI, Foster City, CA, USA). First, mRNA was extracted from cell lysate. Second, cDNA was synthesized with a Moloney murine leukemia virus high performance reverse transcriptase kit (Epicentre Biotechnologies, Madison, WI, USA) using 1 mg of RNA and 0.2 mg oligo-dT serving as primers in a total volume of 20 mL and diluted to 1/100 fold. Third, 4.5 mL of the cDNA was amplified using 9 mL of the Smart Quant Green Master Mix with dUTP and ROX (Protech, Birmingham, UK) and 0.9 mL of mixed primers and 3.6 mL Q-H2O. During the qPCR, a 125 base pairs fragment of VEGFα-encoding mRNA was amplified from the cDNA. The amplicon was detected by means of fluorescence, using a specific pair of VEGF-a primers: forward primer 5′- CTTGTTCAGAGCGGAG AAAGC-3′ and reverse primer 5′-ACATCTGCAAGTACGTTCGTT-3′. The fluorescence emitted after hybridization to the template DNA was measured. In a separate qPCR, mRNA encoding for hypoxanthine-guanine phosphoribosyl transferase primers: forward primer 5′-GT TAAGCAGTACAGCCCCAAA-3′ and reverse primer 5′-AGGGCATA TCCAACAACAAACTT-3′ was processed as a housekeeping gene. Its product served as a control for RNA and relative quantification. All PCR reactions were performed with hot start activated with 15 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 35 seconds at 72°C, and 2 minutes at 72°C.

For VEGFα protein detection, 10^6 exponentially growing various TCC cells were cultured as usual and treated for 24 hours. They were then pelleted by centrifugation, washed with PBS twice, and resuspended in 1 mL of PBS solution. A 100 μL aliquot of immunofluorescence conjugated mouse antibody raised to VEGFα (1:1000 diluted, Millipore Comp., Billerica, MA, USA) was added and cultured in room temperature for 30 minutes. After being washed with PBS twice, analysis was performed on the fluorescence-activated cell sorting Calibur using CellQuest (BD FACSCalibur, CellQuest Inc., Largo, FL).

In the cytotoxic assay, representative low-grade and high-grade TCC cell lines, TSGH8301 and T24, were exposed to chemotherapeutic drugs. These included doxorubicin, gemcitabine, and cisplatin combined with various concentrations of sunitinib for 48 hours. Cellular viability was detected using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In brief, MBT-2 cells (2000 per well) in 100 μL culture medium were seeded into 96-well microplates and incubated at 37 °C for 12 hours prior to drug exposure. The plated cell numbers were calculated to keep control cells growing in the exponential phase throughout the 48-hour incubation period. For concurrent treatment, cells were treated with both single chemotherapeutic drugs and sunitinib (each in 100 μL of culture medium) simultaneously and incubated for 48 hours. Then 50 μL of MTT (2 mg/mL in RPMI medium) were added to each well and allowed to react for 3 hours. The blue formazan crystals formed were pelleted to the well bottoms by centrifugation, separated from the supernatant, and dissolved in 100 μL of dimethyl sulfoxide. The optical density was determined by absorbance spectrometry at 560 nm using an enzyme-linked immunosorbent assay reader (Multiskan ex; Thermo Fisher, Waltham, MA, USA). Three separate experiments with triplicate runs in each were performed to obtain mean cell viability. The drug concentrations inhibiting cell growth by 50% (IC50) were determined using Calcusyn1 (Version 1.1.1, 1996; Biosoft Inc., Cambridge, UK).[5]

The combined effects of the chemotherapeutic drug and sunitinib were subjected to an observed and expected ratio. Control experiments replaced the active agent with a drug-free medium. By combining two agents at graded concentrations, numerous combined effects of growth inhibition were obtained and analyzed using observed/expected (O/ E) ratio. A statistical function was used for analysis:

$$f(u)A,B = f(u)A + f(u)B$$

where f(u)A = the fraction unaffected by drug A, f(u)B = the fraction unaffected by drug B, and f(u)A, B = the fraction unaffected by drugs A and B and formula:

$$(\text{A drug and B drug combination survival rate})/[\times(\text{A drug survival rate}) + (\text{B drug survival rate})]$$

O/E ratios < 1, 1–2, and >2 indicate synergism, additivism, and antagonism, respectively. The expected (presumed to be additive or synergistic) and observed survival rates of TCC cells were obtained from three independent drug-combined treatments.[6]

In the animal study, C3H mice (8 weeks old, $n = 10$ in each group) were obtained from the breeding colony of the National Laboratory Animal Center. Prior to initiating the experiment, we acclimatized all mice to a pulverized diet for 3 days. Our experimental protocol was reviewed and approved by the National Defense Medical Center Laboratory Animal Center. MBT-2 cells (1×10^6) in 100 μL PBS were injected into the subcutaneous flank region using a 27-gauge needle. Visible growing tumors established themselves within 2 weeks after inoculation. Gemcitabine (3.8 mg/mL × 8 shots) was injected intraperitoneally twice a week. Sunitinib (0.25 mg/day) was given daily through oral tube feeding in a 3 weeks on and 1 week off regimen similar to clinical usage. The cotreatment group of sunitinib and gemcitabine complied with the same regimen. The day of cell implantation was designated Day 0. The tumors of the treated group and control group were measured daily with vernier calipers in order to measure the long and short dimensions. Body weight was assessed daily after cell injection. After 30 days, the animals were euthanized and the primary tumors in their flanks were excised, weighed, and processed for histopathological examination.

The expression of mRNA, cytotoxicity in MTT assay, and xenografted tumor volume were expressed as mean ± standard deviation. The Mann–Whitney *U* test was used for statistical comparison with differences considered significant at $p < 0.05$.

3. Results

Real-time PCR assay of VEGFα mRNA in various TCC cell lines

As shown in Fig. 1, high-grade TCC cell lines (T24, TCC8701, and TCC 8702) have higher content of VEGFα mRNA than low-grade TCC cell lines (TCC 8301 and TCC 9201) with 3–6 fold increased changes ($p < 0.01$).