

Benzyl isothiocyanate suppresses IGF1R, FGFR3 and mTOR expression by upregulation of miR-99a-5p in human bladder cancer cells

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Abstract. Benzyl isothiocyanate (BITC) is known for its pharmacological properties against malignant neoplasm, including bladder cancer (BC). The current study investigated microRNAs (miRNA or miR) expression profiles with an emphasis on the role of miR-99a-5p in BITC-treated BC cells. A quantitative polymerase chain reaction (qPCR) microarray containing 79 aberrantly expressed miRNAs in BC was used to detect miRNA expression in BITC-treated cells. Several dysregulated miRNAs were identified and further confirmed using miRNA stem-loop reverse transcription (RT)-qPCR in 5637 cells. Insulin-like growth factor 1 receptor (IGF1R), fibroblast growth factor receptor 3 (FGFR3) and mammalian target of rapamycin (mTOR) expression were determined by RT-qPCR and western blotting. Cell viability was evaluated using WST-1 reagent and apoptosis was monitored by determining the levels of cleaved-poly ADP-ribose polymerase and cleaved-caspase-3. BITC treatment significantly upregulated miR-99a-5p levels in a dose-dependent manner. miR-99a-5p overexpression decreased IGF1R, mTOR and FGFR3 expression, predicted targets of miR-99a-5p. In addition, antisense miR-99a-5p sequences inhibited BITC-induced

miR-99a-5p overexpression, resulting in the restoration of protein expression and decreased cell viability. The current study identified multiple miRNAs responsive to BITC treatment, including miR-99a-5p. In addition, the induction of miR-99a-5p decreased IGF1R, mTOR and FGFR3 expression in BITC-treated BC cells. The current study provided novel insight into the antitumor mechanism by which BITC restores miR-99a-5p expression and decreases cancer cell survival.

Introduction

Bladder cancer (BC) is one of most common malignancies in urinary tract worldwide (1). A total of ~80% of patients with BC are diagnosed with non-muscle invasive BC (NMIBC) (2). The standard treatment for NMIBC is transurethral resection (TUR) (3). Recurrence of NMIBC following TUR is 60-80% (4). Recurrence is attributed to incomplete resection, growth of microscopic tumors, reimplantation of tumor cells or new tumor formation (5). Intravesical therapy with Bacillus Calmette-Geurin (BCG) vaccine or other chemotherapeutic agents, including mitomycin C, are used to prevent or delay recurrence following TUR (6). However, 20-40% of patients respond poorly to these treatments (7) and new therapeutic modalities to prevent high recurrence rates are in a demand.

Benzyl isothiocyanate (BITC) is of the ITC family, which exerts anticancer activity by apoptosis induction in BC cells and inhibiting chemical-induced cancer in animal models (8). A recent study has revealed that BITC induces autophagic cell death in breast cancer (9). In addition, BITC treatment induces apoptosis and autophagy via inhibiting the mammalian target of rapamycin (mTOR) signaling pathway in prostate cancer cells (10). BITC has been reported to inhibit growth of pancreatic cancer cells through manipulating microRNA (miRNA or miR) expression. It is of interest to elucidate the mechanism detailing the anticancer effect of BITC in BC.

miRNAs are small noncoding RNAs (~20-24 nucleotides) that regulate target gene expression through translational blockage or mRNA degradation (11). Increasing studies have reported that miRNAs serve important roles in regulating tumor

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Abbreviations: BITC, benzyl isothiocyanate; BC, bladder cancer; miRNAs, microRNAs; IGF1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; FGFR3, fibroblast growth factor receptor 3

Key words: apoptosis, fibroblast growth factor receptor 3, insulin-like growth factor 1 receptor, microRNA-99a, mammalian target of rapamycin, urinary bladder neoplasms

formation and progression (12). Numerous anticancer agents have been suggested to exert cell toxicity through manipulating miRNA expression (13). A recent study has reported that in patients with BC, miR-99a-5p is downregulated in cancerous tissues (14). The miR-99 family is known to be involved in the mTOR signaling pathway of other cancers (15,16).

The present study focused on the association of miRNA with BITC-induced inhibitory effects in BC. It was hypothesized that BITC inhibited BC cell growth through altering the expression of certain miRNAs. miRNA expression profiles were explored in response to BITC treatment using a miRNA microarray approach. The target genes of miR-99a and downstream effectors were further investigated.

Materials and methods

Chemicals. All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). BITC (purity, ~98%) was prepared as described previously (10).

Cell culture and transfection. Human BC cell lines RT4 (cat. no. HTB-2), 5637 (cat. no. HTB-9), HT1376 (cat. no. CRL-1472), HT1197 (cat. no. CRL-1473), T24 (cat. no. HTB-4) and human-ureter-sumian-virus-40 transformed immortalized epithelial cell line SV-HUC-1 (cat. no. CRL-9520) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely checked for mycoplasma contamination using a polymerase chain reaction (PCR)-based method as described previously (17). RT4 cells were cultured in McCoy's 5A medium, HT1376 and HT1397 cells were maintained in Minimum essential medium, 5637 and T24 cells were cultured in RPMI-1640 and SV-HUC-1 cells were cultured in F12 medium; all media (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were supplemented with 10% fetal bovine serum and essential supplements (Thermo Fisher Scientific, Inc.).

Cells at 70-80% confluence were transfected with below described plasmids in 6-well plates or 10-cm dishes for 24 h prior to treatment with BITC (20 μ M) for 24 h. Transfection of 1 μ g/ml plasmid was performed using a polymer-based transfection reagent (Ultra293; GeneDireX, Inc., Taipei, Taiwan) according to the manufacturer's instructions and transfection efficiency was evaluated by reverse transcription-quantitative (RT-q) PCR.

Profiling of miRNAs expression using a BC miRNA RT-qPCR array. T24 bladder cancer cells were incubated with BITC (20 μ M) for 24 h. The miProfile™ Human BC miRNA qPCR array (cat. no. QM-018; GeneCopoeia, Inc., Rockville, MD, USA), which is able to profile 79 aberrantly expressed miRNAs most relevant to BC, was used to identify miRNA responses to BITC treatment of BC cells. Total RNA from control or BITC-treated 5637 cells was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and the quality and concentration of RNA was determined using a NanoDrop2000 (Thermo Fisher Scientific, Inc.). miRNAs were reverse-transcribed (37°C; 60 min) from 2.5 μ g of total RNA using poly-A polymerase with an oligo(dT) adaptor from the All-in-One™ miRNA First Strand

cDNA Synthesis kit provided with the miRNA qPCR array (GeneCopoeia, Inc.). The qPCR array was performed in 20 μ l reactions containing 1 μ l RT product using the SYBR-Green (cat. no. KK4600; Kapa Biosystems; Roche Diagnostics, Indianapolis, IN, USA) detection on a StepOne Plus instrument (Thermo Fisher Scientific, Inc.). Primers for the miProfile™ human bladder cancer miRNA qPCR arrays were provided with the kit. The following protocol was used: 95°C for 3 min; followed by 40 cycles of 95°C for 3 sec and 60°C for 20 sec; melting curves were recorded between 60-95°C with using 0.1°C/sec as a heat ramp and storage at 4°C. Data was analyzed using All-in-One™ qPCR Primer Array Data Analysis software provided by GeneCopoeia, Inc. and the $2^{-\Delta\Delta C_q}$ method was applied for quantification (18). Small nucleolar RNA U43 (SNORD43) was used as control.

Detection of miRNAs expression by stem-loop RT-qPCR. 5637 bladder cancer cells were incubated with BITC (10 μ M) for 24 h. Total RNA was extracted and miR-99a-5p, miR-133b-5p, miR-30a-3p, miR-30a-5p, miR-125b-5p and miR-195-5p expression was determined by stem-loop RT-qPCR according to a previously published protocol (19). Stem-loop RT primers, universal reverse primer and miRNA specific forward primers are listed in Table I. miRNA was reverse transcribed into cDNA using the miRNA stem loop-RT primers and TaqMan™ MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). miRNAs quantification was performed using the StepOne Plus instrument (Thermo Fisher Scientific, Inc.), with universal reverse primer and miRNA specific forward primers. The Universal ProbeLibrary probe #21 (UPL21) hydrolysis probe had the following sequence: 5'-T+G+G+C+T+C+TG-3', where '+' identifies a unique nucleotide chemistry (Locked Nucleic Acid). SNORD43 was used as loading control. The following protocol was used: 95°C for 5 min; followed by 40 cycles of 95°C for 5 sec, 60°C for 10 sec and 72°C for 1 sec with 0.2°C/sec heating and storage at 4°C.

Construction of miR expression and reporter vectors. The miR-99a-5p expression vector (pSM-99a-5p) was constructed by annealing a paired oligonucleotides consisting of the mature miR-99a sequence (oligonucleotide 1, 5'-TGCTGAACCCGTA GATCCGATCTTGTGGTTTTGGCCACTGACTGACCACA AGATGATCTACGGGTT-3'; and oligonucleotide 2, 5'-CCT GAACCCGTAGATCATCTTGTGGTTCAGTCAGTGGCCAA ACCACAAGATCGGATCTACGGGTTTC-3') and cloned into a small-RNA expression vector (pSM; cat. no. 19170; Addgene, Inc., Cambridge, MA, USA) as previously described (20). The concept of a miRNA sponge targeting miRNA and attenuating its function has been well established (21,22). Following a previous study describing the establishment of a let-7 sponge (23), the synthesized double strand oligonucleotides containing 3 repeats of matured miR-99a-5p antisense sequences were inserted to pmiR-GLO (Promega Corporation, Madison, WI, USA) generating a positive reporter construct (pmiR-GLO-99a-5p-PTS). 5637 and T24 cells (5×10^4 cells/well) at 7-80% confluence were seeded into 24-well plates and transfected with pmiR-GLO-99a-5p-PTS or empty control (pmiR-GLO; 1 μ g/ml) using a polymer-based transfection reagent (Ultra293; GeneDireX, Inc.) according to the manufacturer's instructions. Cells were treated with BITC (20 μ M) for 24 h post-transfection. Following

Table I. Oligonucleotides and probe used for stem-loop RT-qPCR analysis.

A, Stem-Loop RT	
Name	Sequence (5'-3')
hsa-miR-99a-5p_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCACAAG
hsa-miR-133b-5p_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAGTAGCTG
hsa-miR-30a-3p_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGCTGCA
hsa-miR-30a-5p_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCTTCCA
hsa-miR-125b-5p_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAAGTACAA
hsa-miR-195-5p_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGCCAAT
SNORD43_RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAATCAG
B, qPCR	
Name	Sequence (5'-3')
hsa-miR-99a-5p_F	GTGAACCCGTAGATCCGAT
hsa-miR-133b-5p_F	GGGTTTGGTCCCCTTCAAC
hsa-miR-30a-3p_F	GTGCTTTCAGTCGGATGTT
hsa-miR-30a-5p_F	GGGTGTAACATCCTCGAC
hsa-miR-125b-5p_F	GTGTCCTGAGACCTAAC
hsa-miR-195-5p_F	GGGGTAGCAGCACAGAAAT
SNORD43_F	GTGAACCTATTGACGGGCG
Universal reverse primer	GTGCAGGGTCCGAGGT

miR, micro RNA; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; F, forward; SNORD43, small nucleolar RNA U43.

further 24 h, the activities of Firefly and *Renilla* luciferase were detected using Dual-luciferase kit (Promega Corporation). Relative protein levels were expressed as Firefly/*Renilla* luciferase.

Detection of IGF1R, FGFR3 and mTOR expression. T24 and 5637 bladder cancer cells seeded in 6-well plates (3x10⁵ cells/well) were transfected with pSM-99a-5p, pmiR-GLO-99a-5p-PTS or control vectors (1 µg/ml) for 24 h using a polymer-based transfection reagent (Ultra293; GeneDireX, Inc.). Transfection efficiency was evaluated by RT-qPCR and luciferase activity assay as previously described (24). At 24 h post-transfection, transfected cells were incubated with BITC (20 µM) for 24 h. Cells were harvested and lysed by radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich; Merck KGaA). Total proteins from BITC-treated cells transfected with pSM-99a-5p, pmiR-GLO-99a-5p-PTS or control vectors were collected from the lysate and subjected to the detection of insulin-like growth factor 1 receptor (IGF1R), fibroblast growth factor receptor 3 (FGFR3) and mTOR by western blot as described previously (10).

Cell viability assays and detection of apoptosis. Cell viability was determined in bladder cancer cells transfected with pSM-99a-5p, pmiR-GLO-99a-5p-PTS or control vectors treated with BITC (20 µM) for 24 h using WST-1 reagent (Roche Diagnostics GmbH, Mannheim, Germany) as previously described (25). The induction of apoptosis in BITC-treated

cells was determined by assessment of cleaved (c-) poly ADP-ribose polymerase (PARP) and c-caspase-3 by western blotting.

Western blot analysis. Protein levels of cells treated with 10 or 20 µM BITC for 24 h were examined using western blot analysis as described for the immunoblotting for IGF1R, FGFR3 and mTOR3. Antibodies against IGF1R (ab39675), FGFR3 (ab133644), mTOR (ab87540) were purchased from Abcam (Cambridge, UK). c-PARP (#9532), c-caspase-3 (#9661) and β-actin (#4967) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Resolved proteins were transferred to polyvinyl difluoride membranes. Blots were blocked with 5% nonfat milk for 1 h at room temperature followed by incubation with primary antibodies (1:1,000) for 1 h at room temperature. Following washes with TBST (3x), blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:1,000; cat. no. GTX213110-01) or anti-mouse secondary antibody (1:1,000; cat. no. GTX213111-01) (both from GeneTex, Inc., Irvine, CA, USA) for 1 h at room temperature followed by TBST washes (3x). Blots were visualized using an enhanced chemiluminescence detection system (Amersham; GE Healthcare, Chicago, IL, USA) according to the manufacturer's instruction. Densitometry was performed using ImageJ software 1.49v (National Institutes of Health, Bethesda, MD, USA). β-actin was used as internal control. Results are expressed as the mean ± standard deviation (SD) of three independent experiments.

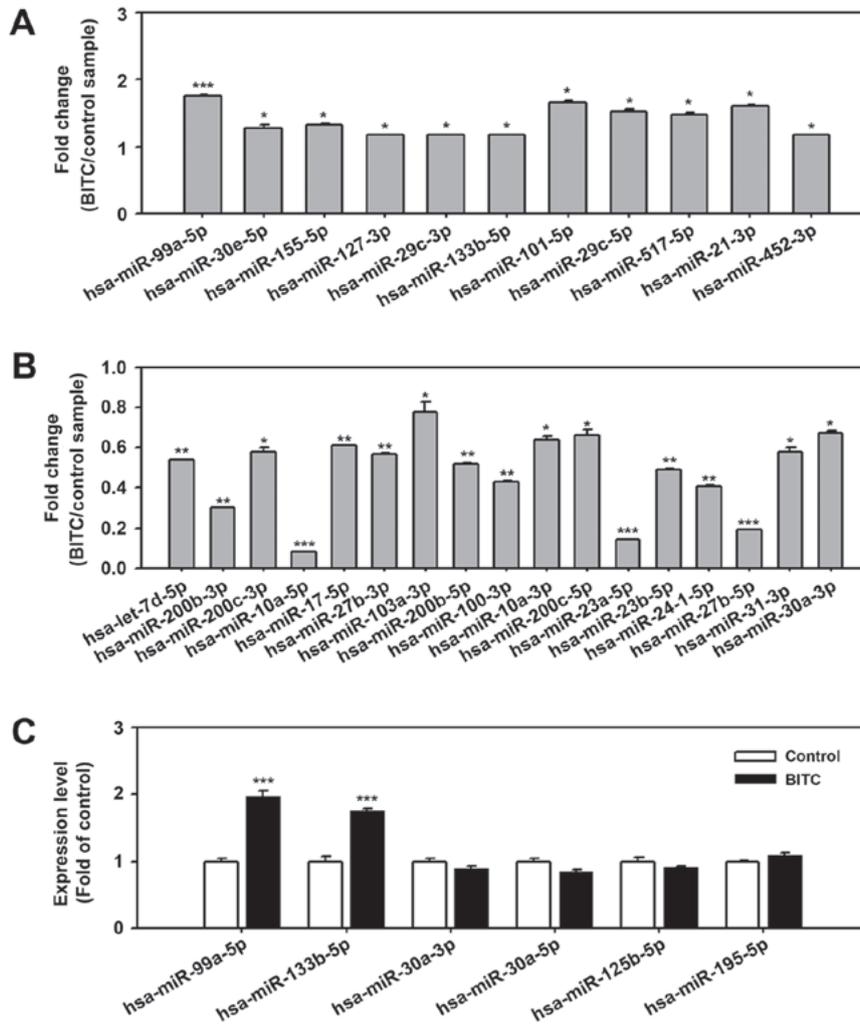


Figure 1. BITC treatment affects miR expression in 5637 cells. Expression levels of 79 bladder cancer-associated miRs were detected using a commercial miR qPCR array. Significantly (A) upregulated and (B) downregulated miRs identified in BITC-treated cells. (C) Expression of selected miRs detected by miR stem-loop reverse transcription-qPCR for validation. Expression changes were normalized to the control and are presented as fold change. The results are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001 vs. control normalized to 1 (not presented). BITC, benzyl isothiocyanate; miR, microRNA; qPCR, quantitative polymerase chain reaction.

Statistical analysis. All experiments were performed ≥ 3 times, each in triplicate and data are presented as the mean \pm SD. Statistical analysis between two samples were performed using Student's t-test. Multiple group comparisons were performed using one-way analysis of variance with Bonferroni's post-hoc tests. P <0.05 was considered to indicate a statistically significant difference.

Results

BITC upregulates miR-99a-5p expression in BC. Dysregulation of miRNA has been reported in BC tissues, with 19 up- and 11 downregulated miRNAs (14). To investigate the effect on BITC treatment on miRNA expression in BC cells, expression profiles were determined using a miRNA qPCR array containing 79 aberrantly expressed miRNAs in BC. The results suggested that 11 miRNAs were significantly upregulated in BITC-treated BC cells compared with untreated control cells, including miR-30e-5p, miR-155-5p, miR-127-3p, miR-29c-3p, miR-133b-5p, miR-101-5p, miR-29c-5p, miR-517-5p, miR-21-3p, miR-452-3p (all P <0.05) and miR-99a-5p

(P <0.001; Fig. 1A). A total of 17 miRNAs were significantly downregulated in BITC-treated 5637 cells compared with untreated control cells including miR-200c-3p, miR-103a-3p, miR-10a-3p, miR-200c-5p, miR-31-3p, miR-30a-3p (all P <0.05), let-7d-5p, miR-200b-3p, miR-17-5p, miR-27b-3p, miR-200b-5p, miR-100-3p, miR-23b-5p, miR-24-1-5p (all P <0.01), miR-10a-5p, miR-23a-5p and miR-27b-5p (all P <0.001; Fig. 1B). BITC has been proposed to induce autophagy in breast cancer and prostate cancer cells (9,10). To explore the correlation between dysregulated miRNAs and autophagy regulation, miRNAs reported to regulate the autophagy pathway, including miR-99a-5p (24), miR-133b-5p (26), miR-30a-3p (27), miR-30a-5p (28), miR-125b-5p (29) and miR-195-5p (30) were further validated by miRNA stem-loop RT-qPCR. Following validation by stem-loop RT-qPCR, data confirmed that miR-99a-5p and miR-133b-5p expression was significantly upregulated in 5637 cells treated with BITC compared with the normal control (P <0.001; Fig. 1C). However, expression of miR-30a-3p, miR-30a-5p, miR-125b-5p and miR-195-5p were not significantly affected by exposure to BITC.

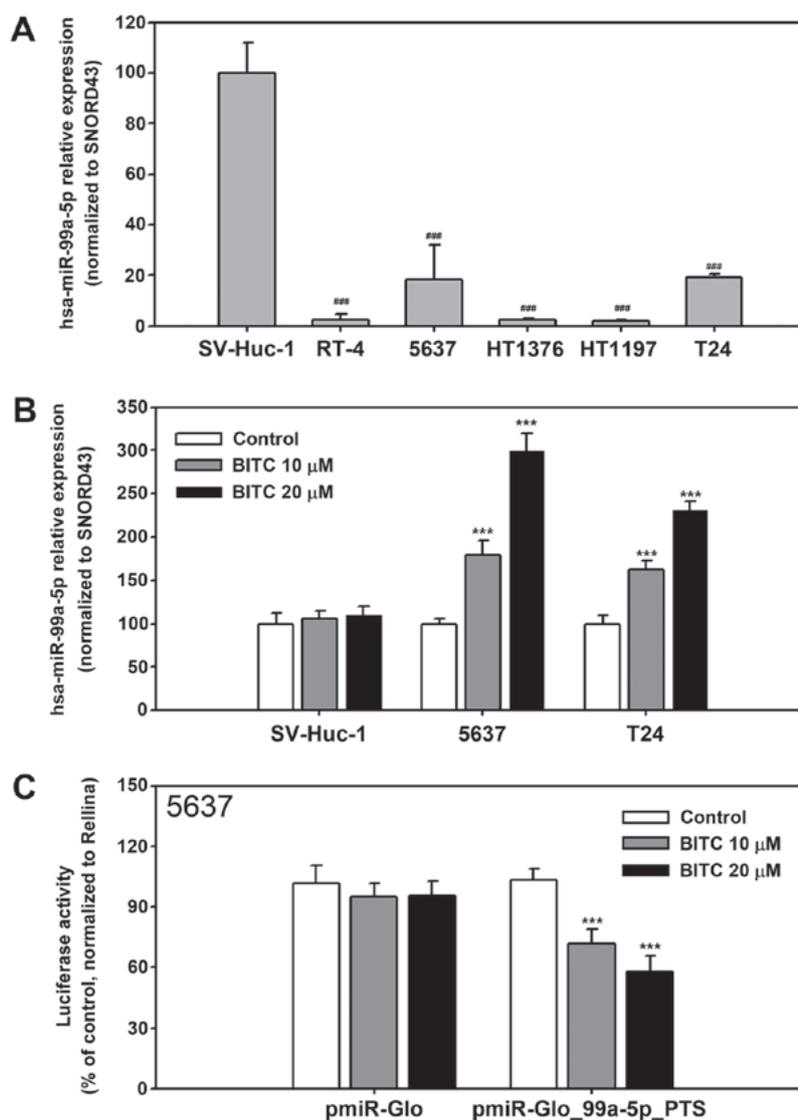


Figure 2. miR-99a-5p is upregulated in BITC-treated BC cells. (A) miR-99a-5p expression in human BC and immortalized urothelial cells. $^{***}P < 0.001$ vs. SV-Huc-1. (B) miR-99a-5p expression in 5637, T24 and SV-Huc-1 cells treated with 10 or 20 μ M BITC. (C) Luciferase activity of 5637 cells treated with 10 or 20 μ M BITC transfected with pmiR-Glo-99a-5p-PTS or empty vector. Results are presented as the mean \pm standard deviation. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control. BITC, benzyl isothiocyanate; miR, microRNA; pmiR-Glo, empty luciferase vector; pmiR-Glo-99a-5p-PTS, vector containing miR-99a-5p antisense sequences.

To confirm that BITC induced miR-99a-5p expression, miR-99a-5p expression in untreated SV-Huc-1, RT4, 5637, HT1376, HT1197 and T24 cells was determined using stem-loop miRNA RT-qPCR. As presented in Fig. 2A, miR-99a-5p expression was significantly downregulated in all BC cells compared with the SV-Huc-1 normal cells ($P < 0.001$). 5637 and T24 cells were used in following based on higher transfection efficiencies compared with the other cell lines. BITC treatment increased miR-99a-5p expression in 5637 and T24 cells compared with the untreated cells ($P < 0.001$); no significant changes were observed for SV-Huc-1 cells ($P > 0.05$; Fig. 2B). To further evaluate the effect of BITC treatment on miR-99a-5p expression, luciferase assays were performed using pmiR-Glo-99a-5p-PTS, which is able to bind miR-99a-5p. The reporter construct was transfected into 5637 cells and cells were treated with BITC for 24 h post transfection. The results demonstrated a significant dose-dependent decrease in luciferase activity upon BITC treatment compared

with the untreated control, indicating miR-99a-5p upregulation in BITC-treated cells ($P < 0.001$; Fig. 2C).

miR-99a-5p overexpression and BITC treatment decrease IGF1R, FGFR3 and mTOR expression in BC cells. Changes in the expression of target genes of miR-99a-5p were evaluated following miR-99a-5p overexpression and BITC treatment of BC cells. IGF1R, mTOR and FGFR3 expression was determined in 5637 and T24 cells transfected with a miR-99a-5p overexpressing vector. IGF1R, mTOR and FGFR3 mRNA was significantly decreased in pSM-99a-5p transfected 5637 and T24 cells compared with the empty vector control ($P < 0.01$, $P < 0.001$ and $P < 0.001$, respectively; Fig. 3A). Protein expression levels were also significantly decreased in the miR-99a-5p overexpression samples compared with the control ($P < 0.001$ for 5637 cells and $P < 0.01$, $P < 0.001$ and $P < 0.01$ for IGF1R, mTOR and FGFR3 in T24, respectively; Fig. 3B). To investigate the expression of IGF1R, mTOR and FGFR3

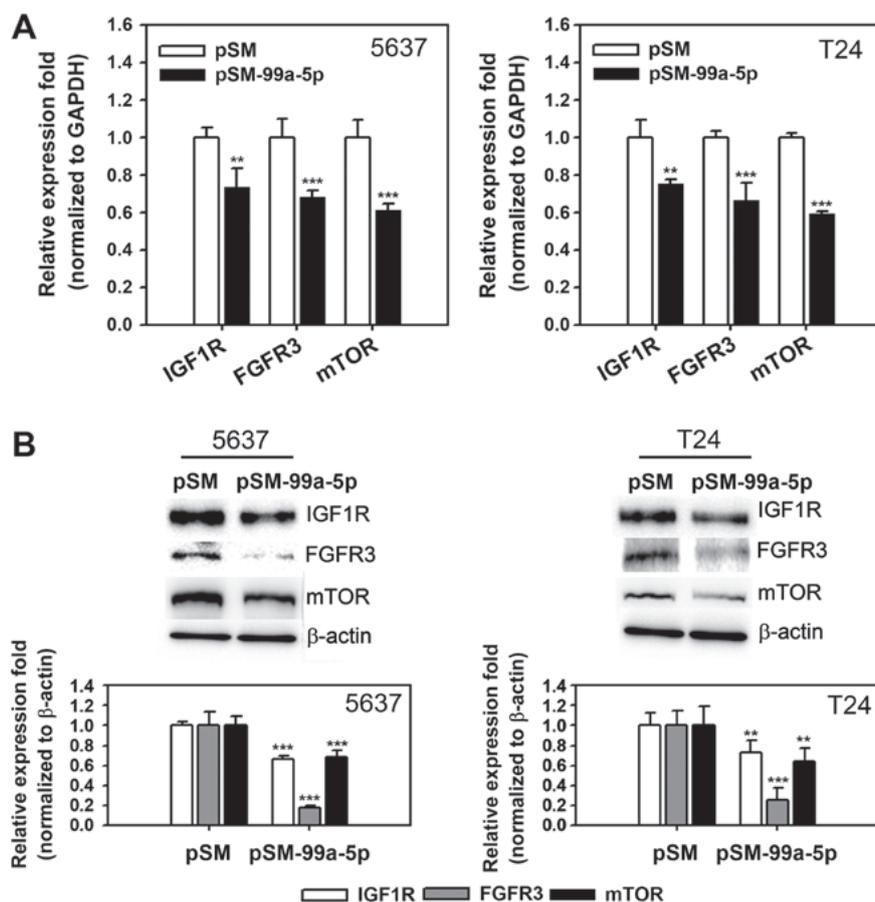


Figure 3. miR-99a-5p overexpression decreases IGF1R, mTOR and FGFR3 expression. Bladder cancer 5637 and T24 cells were transfected with pSM-99a-5p or an empty vector control and (A) mRNA and (B) protein expression of IGF1R, mTOR and FGFR3 were detected by reverse transcription-quantitative polymerase chain reaction and western blot assays, respectively. β -actin was used as internal control. Results are representative of three independent experiments and are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001 vs. pSM. miR, microRNA; IGF1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; FGFR3, fibroblast growth factor receptor 3.

in BITC-treated cells, mRNA and protein expression was determined in 5637 and T24 cells treated with BITC. As presented in Fig. 4A, BITC treatment (20 μ M) resulted in decreased mRNA expression of IGF1R, mTOR and FGFR3 compared with the untreated control (P <0.001 for 5637 and P <0.001, P <0.01 and P <0.001 for IGF1R, mTOR, and FGFR3 in T24, respectively). Protein expression was significantly decreased in BITC-treated cells in a dose-dependent manner compared with the untreated control (P <0.01 and P <0.001 for 10 and 20 μ M BITC, respectively; Fig. 4B). The results indicated that miR-99a-5p overexpression and BITC treatment inhibited the expression of IGF1R, mTOR and FGFR3 pro-survival proteins in BC cells.

miR-99a-5p inhibition attenuates BITC-induced IGF1R, FGFR3 and mTOR downregulation and decreased cell viability. A previous study by the authors focused on pmiR-Glo-99a-5p-PTS, which expressed antisense miR-99a-5p and exhibited inhibitory effects on miR-99a-5p function (24). To confirm that IGF1R, mTOR and FGFR3 inhibition was mediated by miR-99a-5p upregulation through BITC treatment, experiments using pmiR-Glo-99a-5p-PTS acting as competitors to BITC-induced miR-99a-5p expression were performed. IGF1R, mTOR and FGFR3 expression was detected in transfected cells that received 24 h BITC treatment

(20 μ M). As presented in Fig. 5A, overexpression of miR-99a-5p induced IGF1R and mTOR expression in 5637 cell (P <0.001 and P <0.01, respectively). Furthermore, IGF1R, mTOR and FGFR3 protein expression downregulation in BITC-treated cells was significantly reversed by antisense miR-99a-5p expressing, BITC-treated cells (P <0.001, P <0.05 and P <0.001, respectively). Effects of miR-99a-5p inhibition on the viability of BITC-treated cells were further evaluated. Cell viability was significantly decreased in BITC-treated cells compared with the untreated controls (P <0.001; Fig. 5B). Inhibition of miR-99a-5p significantly reversed the BITC-induced viability decrease in 5637 and T24 cells (P <0.01 and P <0.05, respectively; Fig. 5B). The results suggested that BITC treatment suppressed IGF1R, mTOR and FGFR3 expression by upregulating miR-99a-5p levels. However, there may be further effectors, in addition to miR-99a-5p, that contributed to BITC-induced cytotoxicity in BC cells.

Effects of miR-99a-5p overexpression are enhanced by BITC treatment in BC cells. It was evaluated if combination of miR-99a-5p overexpression and BITC treatment enhanced cell death in BC cells compared with the single treatments. As presented in Fig. 6A, BITC treatment (10 μ M) and miR-99a-5p overexpression alone significantly decreased cell viability in 5637 and T24 cells compared with the untreated cells (P <0.001).

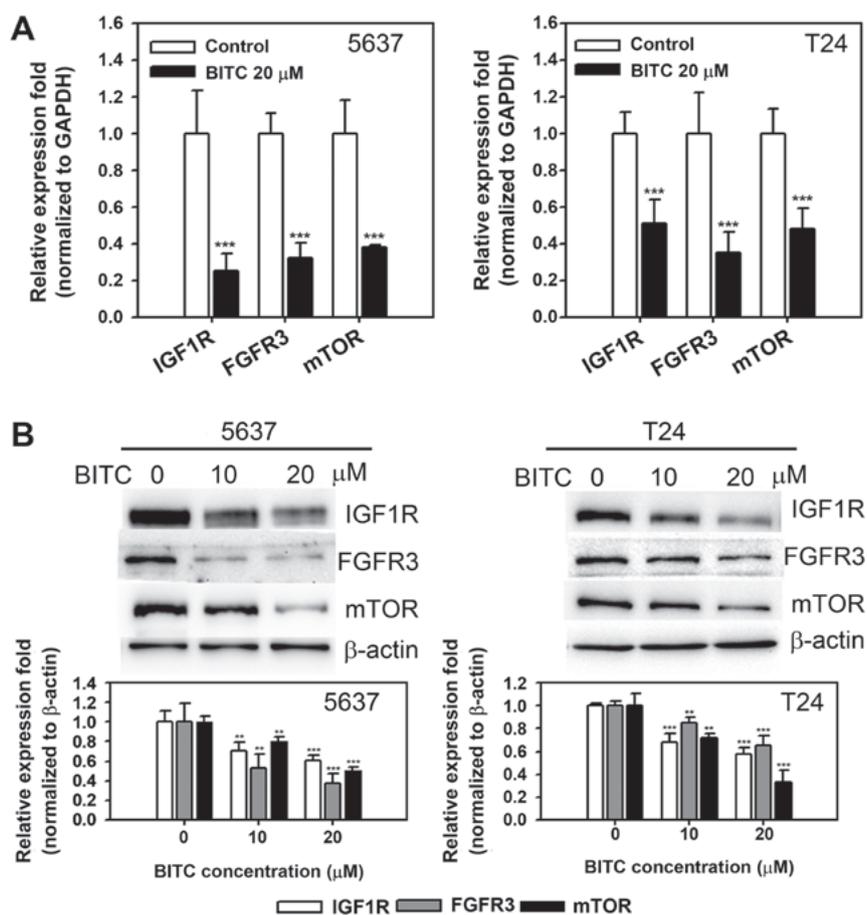


Figure 4. BITC treatment decreases IGF1R, mTOR, and FGFR3 expression. (A) IGF1R, mTOR and FGFR3 mRNA expression in BITC-treated (20 μ M) 5637 and T24 cells determined by reverse transcription-quantitative polymerase chain reaction. (B) IGF1R, mTOR and FGFR3 protein expression in BITC-treated (10 and 20 μ M) 5637 and T24 cells determined by western blotting. β -actin was used as internal control. Results are representative of three independent experiments and are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001 vs. control. BITC, benzyl isothiocyanate; IGF1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; FGFR3, fibroblast growth factor receptor 3.

Cell viability was further significantly decreased when combining the two treatments in 5637 and T24 cells compared with miR-99a-5p overexpression alone (P <0.001). Apoptosis induction was evaluated by determining the cleavage of PARP and caspase-3. Cleaved protein levels were significantly increased in miR-99a-5p overexpressing or BITC-treated cells compared with the untreated cells (P <0.01 and P <0.001, respectively; Fig. 6B). The combination of miR-99a-5p overexpression and BITC treatment further significantly increased the c-PARP and c-caspase-3 levels compared with the BITC treatment group (P <0.001). The results suggested that miR-99a-5p overexpression enhances the effects of BITC in BC cells.

Discussion

BC is one of the leading causes of cancer-associated mortality worldwide (31). Novel therapeutic approaches preventing the recurrence or improving the survival of patients with BC are desirable. In the current study, it was described that BITC inhibited IGF1R, mTOR and FGFR3 expression through upregulation of the tumor suppressing miR-99a-5p. The results further demonstrated that elevation of miR-99a-5p levels enhanced BITC-induced cytotoxicity in BC cells. Increasing evidence has highlighted anti-cancer activities of BITC in a variety of tumor cell lines and in rodent animal models (32).

BITC is suggested to inhibit growth, induce apoptosis and G2/M phase cell cycle arrest in BC cells (33). The current study suggested that BITC decreased 5637 and T24 cell viability by induction of apoptosis.

miRNAs are important regulatory components in tumorigenesis and several miRNAs are considered as therapeutic targets in BC (34). Tumor suppressing activities of miR-99a-5p have been investigated in various types of cancer; miR-99a-5p exerts anti-metastasis abilities in human non-small cell lung cancer cells by inhibiting protein kinase B1 and in oral cancer by inhibiting myotubularin-related protein 3 expression (35,36). In mammary gland cells miR-99a-5p modulates transforming growth factor- β induced epithelial to mesenchymal plasticity (37). mTOR targeting and inhibition of cell proliferation or induction of apoptosis have been demonstrated in anaplastic thyroid (15), breast (16) and cervical cancer (38). miR-99a-5p controls IGF1R and mTOR expression in human hepatocellular carcinoma (39-41) and has been reported to be downregulated in human BC, leading to the upregulation of FGFR3 (14,42). IGF1R, FGFR3 and mTOR are known anti-apoptotic regulators (39-41). The results of the current study indicated antitumor effects of miR-99a-5p by inducing apoptosis in BC. A previous study reported that miR-99a-5p is downregulated in bladder urothelial carcinoma tissue compared with normal tissue (14).

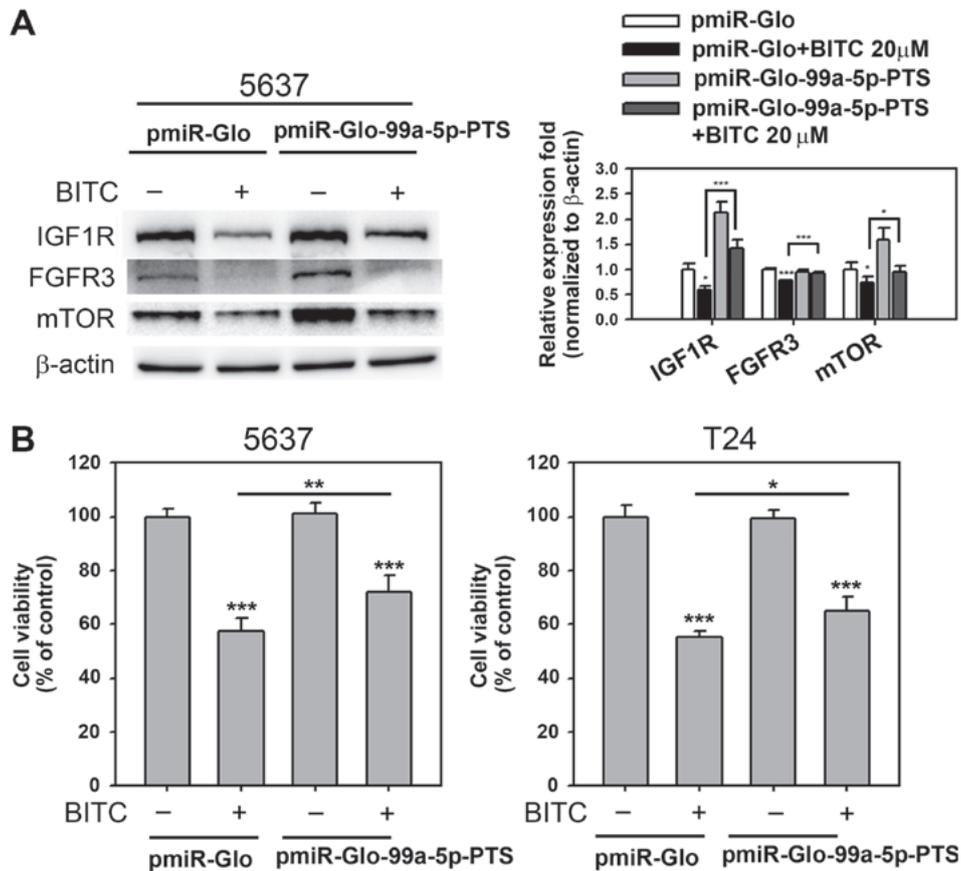


Figure 5. Inhibition of BITC-induced miR-99a-5p expression attenuates protein expression and decreases cell viability. Bladder cancer cells transfected with pmiR-Glo-99a-5p-PTS or empty vector were treated with BITC (20 μ M). (A) IGF1R, mTOR and FGFR3 protein expression in 5637 cells treated as detailed above determined by western blotting. β -actin was used as internal control. Results are representative of three independent experiments. (B) Cell viability of 5637 and T24 cells treated as indicated was determined using WST-1 reagent. Results are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001 vs. pmiR-Glo. BITC, benzyl isothiocyanate; miR, microRNA; pmiR-Glo, empty luciferase vector; pmiR-Glo-99a-5p-PTS, vector containing miR-99a-5p antisense sequences; IGF1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; FGFR3, fibroblast growth factor receptor 3.

Furthermore, expression levels and prognostic roles of IGF-1R, mTOR and FGFR3 have previously been reported for BC (43-45). The small number of patients with BC that participated in the current study describes a limitation and IGF1R, mTOR and FGFR3 expression will be investigated further in future experiments.

miR-99a-5p exhibits anticancer activity in various cancer types and BITC has been reported to induce apoptosis in BC cells (46). The current study demonstrated that BITC induced miR-99a-5p expression in BC cells but not normal human urothelial cells. Furthermore, it was suggested that miR-99a-5p may be involved in the regulation of IGF1R, mTOR and FGFR3 in BITC-treated BC cells. A previous review has reported the application of a miRNA sponge, containing multiple miRNA binding sites, in miRNA inhibition (21,22). The results of the current study confirmed that overexpression of miR-99a-5p sponge reversed IGF1R, mTOR and FGFR3 protein expression downregulation in BITC-treated cells. A previous review has addressed the antitumor mechanisms exerted by various ITCs (47). The report proposed that production of reactive oxygen species (ROS) is the common link of ITCs in apoptosis induction. In addition, normal cells exhibit increasing resistance to ROS production and apoptosis induced by

ITCs, suggesting that the induction of miR-99a-5p by BITC treatment in BC cells may contribute to ROS production. These suggestions require to be verified in future experiments.

Various miRNAs that promote cancer cell death are recognized as potential novel anti-cancer agents in various cancers, including BC (34). miR-34a is downregulated during cancer progression and considered a novel target for treating various types of cancer (48). miRNAs rapidly degrade in circulating blood, making an oral or intravenous administration ideal for delivery (49). Bladder instillation had been routinely performed in clinic using chemotherapeutic agents, including BCG or mitomycin C to prevent recurrence of BC (50). Intravesical therapy by delivery of small non-coding RNA is an alternative approach to overcome drug delivery system problems and successfully deliver siRNAs *in vivo* (51). Therapeutic effects of miR-582-5p and -3p (52) and miR-145 (53) administered intravesically in a mouse orthotopic model suggest promising effects against BC. N-acetylcysteine (NAC)-conjugated BITC is the major metabolite detected in urine collected from human donors in different studies (54). It remains to be investigated whether NAC-BITC has the ability to induce miR-99a-5p expression. The present study focused on the dysregulation of miRNAs

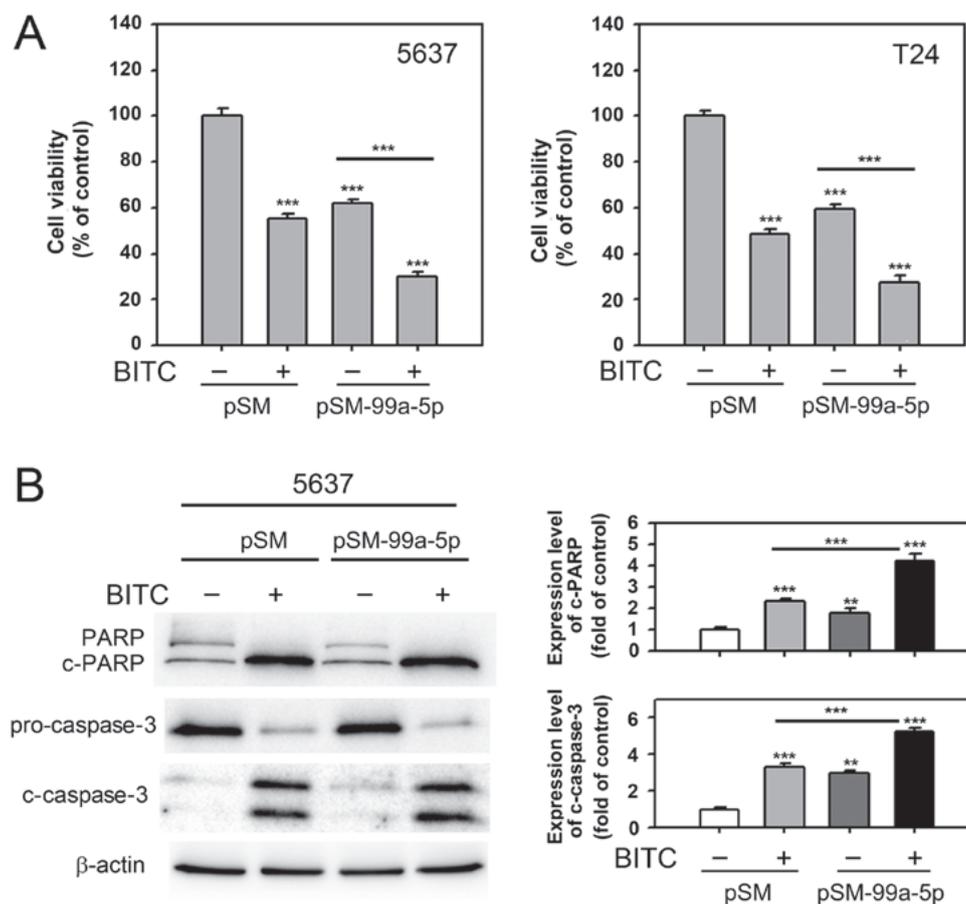


Figure 6. miR-99a-5p overexpression enhances BITC-induced cytotoxicity and apoptosis. Bladder cancer cells transfected with pSM-99a-5p or empty vector were treated with BITC (20 μ M). (A) Cell viability of 5637 and T24 cells with indicated treatment detected by WST-1. (B) Levels of pro-apoptotic marker proteins, c-PARP, PARP, pro-Casp3 and c-Casp3 were detected in 5637 cells with indicated treatment using western blotting. β -actin was used as internal control. Results are representative of three independent experiments and are presented as the mean \pm standard deviation. * P <0.05, ** P <0.01 and *** P <0.001 vs. pSM. BITC, benzyl isothiocyanate; miR, microRNA; c-, cleaved; PARP, poly ADP-ribose polymerase.

following BITC treatment in BC. In initial experiments, miR-99a-5p demonstrated the strongest response to BITC treatment and was selected for further investigation of its role in BC progression. It was demonstrated that ectopic miR-99a-5p expression in combination with BITC treatment decreased cell viability of BC cells compared with either single treatment. The current study suggested that miR-99a-5p may be a novel anticancer agent, alone or combined with other chemotherapeutic agents, and has the potential to inspire future experiments in a preclinical setting.

In this study, it was demonstrated for the first time that BITC treatment inhibited expression of prosurvival proteins IGF1R, mTOR and FGFR3 by upregulation of miR-99a-5p in human BC cells. miR-99a-5p overexpression potentiated the cytotoxicity of BITC in BC cells. Orthotopic animal models using *in vivo* imaging system detection have been widely applied in BC studies (55) and miRNA replacement therapy provides strong preclinical evidence for miRNA-based treatment of cancer (56,57). These preclinical results may shape future experiments studying the effects of miR-99a-5p in BC treatment.

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Availability of data and material

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JFL and TFT conceived and performed the experiments, data interpretation and writing of the manuscript. JFL and TIH designed the study. YCL, HEC and KYC provided the study materials and participate in the interpretation of the experiment data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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