RESEARCH ARTICLE

Potential Mechanisms of Benzyl Isothiocyanate Suppression of Invasion and Angiogenesis by the U87MG Human Glioma Cell Line

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Abstract

Glioma is one of the most common tumors in China and chemotherapy is critical for its treatment. Recent studies showed that benzyl isothiocyanate (BITC) could inhibit the growth of glioma cells, but the mechanisms are not fully understood. This study explored the inhibitory effect of BITC on invasion and angiogenesis of U87MG human glioma cells *in vitro* and *in vivo*, as well as potential mechanisms. It was found that BITC could inhibit invasion and angiogenesis of human glioma U87MG cells by inducing cell cycle arrest at phase G2/M. It also was demonstrated that BITC decreased expression of cyclin B1, p21, MMP-2/9, VE-cadherin, CD44, CXCR4 and MTH1, the activity of the telomerase and PKCζ pathway. Microarray analysis was thus useful to explore the potential target genes related to tumorigenic processes. BITC may play important roles in the inhibition of invasion and angiogenesis of human glioma cells.

Keywords: Glioma cells - benzyl isothiocyanate - invasion- angiogenesis

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Introduction

Glioma is one of the most common cancer worldwide and most patients present with metastasis disease at diagnosis time. Therefore, metastasis management is of great importance to the clinical therapeutics. Metastasis is a complex process, such as loss of cellular adhesion, increased invasiveness, entried to circulation, colonized into new tissue, and developed to metastasis site by cell adhesion molecules, extracellular matrix protein and tumor-related gene (Fidler, 2011).

Recent studies showed that edible cruciferous plants, such as broccoli, cabbage and water celery, could reduce the risk of cancer. Benzyl isothiocyanate (BITC) is an active compound in edible cruciferous plants and it is showed anti-tumor activity in many types of tumor (Zhu et al, 2013). In this study, we focused on BITC and investigated its inhibitory effects on glioma U87 MG cell metastasis potential, and explored effects on U87 cell invasion and angiogenesis and related mechanisms.

Materials and Methods

Cell culture

Human glioma U87MG cell line was cultured in DMEM medium (containing 10% FCS, 100μg/mL streptomycin and 100μg/mL penicillin) with 37°C, 5% CO_2.

Effects of BITC on cell adhesion and invasion of human glioma U87MG cells

Human glioma U87MG cells (2×10^5/well) were cultured in Matrigel-coated 96 well plate and 2μM and 5μM BITC was added for 6h. Then cell were washed by PBS 3 times and cell adhesion rate was measured by MTS assay.

Human glioma U87MG cells (5×10^4/well) were cultured in hydrated Matrigel upper chambers of Transwell insert and 2μM and 5μM BITC was added for 24h. Then lower surface of Transwell insert was fixed by paraformaldehyde and stained with 0.05% crystal violet. Then invaded cells were counted by microscope.

Effects of BITC on cell vasculogenic mimicry (VM) of human glioma U87MG cells

Matrigel were prepared by 1:1 volume ratio of Matrigel and serum free medium. Human glioma U87MG cells (1×10^6) were suspended in 400ul Matrigel and seeded into 24-well plate. The gels were allowed to solidify for 60 minutes at 37°C then added combined medium onto

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each gel. Cultures were maintained for 10 days and the medium was changed.

Effects of BITC on cell cycle of human glioma U87MG cells

Human glioma U87MG cells (5×10^5/well) cultured in 6 well plate with 37°C, 5% CO_2 for 24h and 2μM and 5μM BITC was added for another 24h. Cells were digested, fixed by 70% cold ethanol at 4°C overnight and incubated with PI with 30min in dark at room temperature and cell cycle of tumor cells was measured by flow cytometry.

Effects of BITC on telomerase activity of human glioma U87MG cells

Human glioma U87MG cells (5×10^5/well) were cultured in 6 well plate with 37°C, 5% CO_2 for 24h and 2μM and 5μM BITC was added for another 24h. Telomerase activity of human glioma U87MG cells by ELIASA in accordance with operating manuals.

Effects of BITC on mRNA expresson of tumor-related gene of human glioma U87MG cells

Human glioma U87MG cells (5×10^3/well) were cultured in 6 well plate with 37°C, 5% CO_2 for 24h and 2μM and 5μM BITC was added for another 24h. Total RNA was extracted by Trizol assay. Real-time PCR assays were performed by SYBR green incorporation. GAPDH was used as a normalization. The following primers were used: cyclin B1 (forward) 5'-CTGCCTGGTGAAGAGGAAGC-3' and (reverse) 5'-GAGTGCTAATCTTAGCATGC-3'; p21 (forward) 5'-CACTCCAAACGCCGGCTGATCTTC-3' and (reverse) 5'-TGTAGAGCGGGCCTTTGAGGCCCTC-3'; MMP-2 (forward) 5'-GGCTGGTCAGTGGCTTGGGGTA-3' and (reverse) 5'-AGATCTTCTTCTTCAAGGACCGGTT-3'; MMP-9 (forward) 5'-ACCGTGCCGTGATAGATGAT-3' and (reverse) 5'-AGCCACCAAGAAGATGCTGT-3'; VE-cadherin (forward) 5'-CGGTCAAGTATGGGCAGTTT-3' and (reverse) 5'-CAACTGCTCGTGAATC-3'; CD44 (forward) 5'-TTGATGGACCAATTACCATAACTATTG-3' and (reverse) 5'-CGTTCTGTATTCTCCTTTCTGGACAT-3'; CXCR4 (forward) 5'-CAGAAGAAGCTGAGGAGCATGACA-3' and (reverse) 5'-CTGATGAAGGCCAGGATGAGAACA-3'; MTH1 (forward) 5'-CTCTCCAGCCCTTGTTCAAGTTC-3' and (reverse) 5'-CCTACTCTCTTGGGCTTCATCC-3'; GAPDH (forward) 5'-TGTTGCCATCAATGACCCCTT-3' and (reverse) 5'-CTCCACGACGTACTCAGCG-3'. The PCR condition was as following: denaturation at 95°C for 5 sec, followed by 35 cycles at 95°C for 5 sec and at hybridization 72°C for 60 sec.

Effects of BITC on protein expresson of tumor-related gene of human glioma U87MG cells

Human glioma U87MG cells (5×10^3/well) were cultured in 6 well plate with 37°C, 5% CO_2 for 24h and 2μM and 5μM BITC was added for another 24h. Cells were digested, lysed and total protein was extracted. Total protein was electrophoresis by 12% SDS polyacrylamide gel and transfer the protein to a PVDF membrane. The membrane was incubated at 4°C with 5% skimmed milk overnight. Cyclin B1 antibody (1:500), p21 antibody (1:500), MMP-2 antibody (1:500), MMP-9 antibody (1:500), VE-cadherin antibody (1:500), CD44 antibody (1:500), CXCR4 antibody (1:500) and MTH1 antibody (1:500), and PKCζ antibody (1:400) and β-actin (1:5000) was added and incubated at 4°C overnight. Then IgG labeled with horseradish peroxidase (1:2000) was added and incubated at room temperature for 1h. The protein was visualized with the Phototope HRP Western blot detection system.

Effects of BITC on potential tumor target genes of human glioma U87MG cells

Human glioma U87MG cells (5×10^3/well) were cultured in 6 well plate with 37°C, 5% CO_2 for 24h and 2μM and 5μM BITC was added for another 24h.Total RNA was extracted by RNeasy kit The quality of RNA was determined by Experion Automated Electrophoresis Station and expression of gene and microRNAs was analysed by microarray experiments by Affymetrix GeneChip Human Genome U133 Plus 2.0 in accordance with operating manuals.

Statistical analysis

The data is analyzed by SPSS11.0 statistical software. All data is presented as mean±standard deviation and analyzed by one-way ANOVA. Differences were considered significant at values of $p<0.05$. Each experiment is repeated for three times.

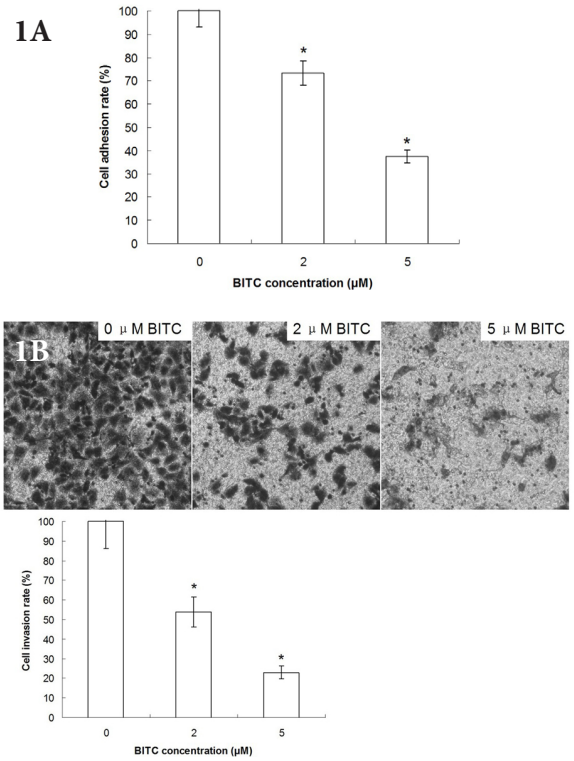

Figure 1. The effect of BITC on Cell Adhesion and Invasion of Human Glioma U87MG Cells. (A) The effect of BITC on adhesion potential of U87MG cells was determined by adhesion assay. Bars mean ± SD. *$P<0.05$, compared with 0 μM group, n=10. **(B)** The effect of BITC on invasion potential of U87MG cells was determined by Transwell assay. Bars mean ± SD. *$p<0.05$, compared with 0 μM group, n=3

Results

BITC suppresses cell adhesion and invasion of human glioma U87MG cells in vitro

As shown by MTS assay, the adhesion potential *in vitro* of U87MG cells was suppressed by 2μM and 5μM BITC treatment for 24h (Figure 1A), meanwhile, the invasion potential *in vitro* of U87MG cells was suppressed by 2μM and 5μM BITC treatment for 24h (Figure 1B).

BITC suppresses cell vasculogenic mimicry (VM) of human glioma U87MG cells

The process by which a vessel is formed from tumor cells is called vasculogenic mimicry (VM), and angiogenesis refers to the unique ability of aggressive tumor cells. To explore whether BITC mediates angiogenesis ability alteration of U87MG cells, we established an *in vitro* model of 3D culture to investigate VM formation. As shown by 3D culture assay, the vasculogenic mimicry *in vitro* of U87MG cells was suppressed by 2μM and 5μM BITC treatment for 24h (Figure 2).

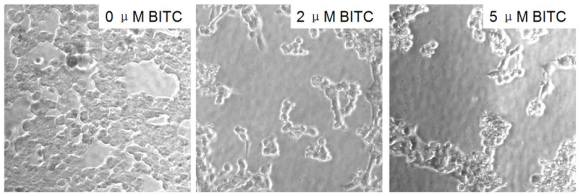

Figure 2. The Effect of BITC on Cell Vasculogenic Mimicry (VM) of Human Glioma U87MG Cells. Matrigel 3D culture was utilized as a well-established *in vitro* model for investigating vasculogenic mimicry. BITC could suppress pipe-like structures formation within the Matrigel medium

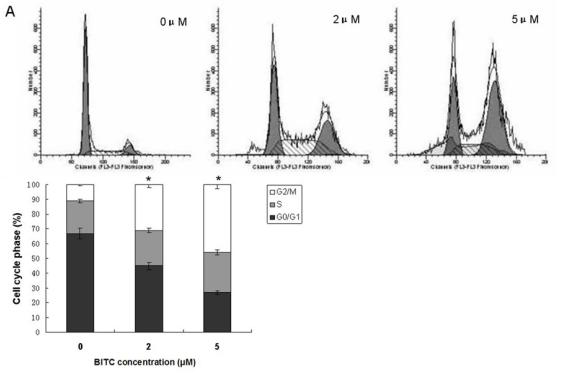

Figure 3. The Effect of BITC on Cell Cycle of Human Glioma U87MG Cells. The effect of BITC on cell cycle of U87MG cells was determined by flow cytometry. Bars mean±SD. *p<0.05, compared with 0 μM group, n=3

Table 1. Tumor-Related Genes Modulated by BITC

Gene	Fold change	Gene	Fold change
CRYAB	3.6	ERI2	-1.1
CLDN1	3.5	TGFB1	-1.4
GADD45	3.5	IFNB2	-1.8
HSPA1B	3.3	BCL2A1	-1.8
PTEN	3.1	ZEB1	-1.9
SCIN	3.1	BCCIP	-2.0
NR2E1	3.0	TWIST	-2.1
TNFAIP3	2.8	hTRT	-2.2
BAG3	2.6	AGPS	-2.3
AXUD1	2.6	MAPK9	-2.5
CDKN2B	2.1	NUDT1	-2.6

BITC suppresses cell cycle of human glioma U87MG cells

As shown by flow cytometry assay, the cell cycle of U87MG cells was arrested on G2/M phase by 2μM and 5μM BITC treatment for 24h (Figure 3).

BITC regulates expresson of tumor-related gene of human glioma U87MG cells

As shown by Figure.4, there was a decreased mRNA and protein expression of cyclin B1, p21, MMP-2/9, VE-cadherin, CD44, CXCR4 and MTH1 and the activity

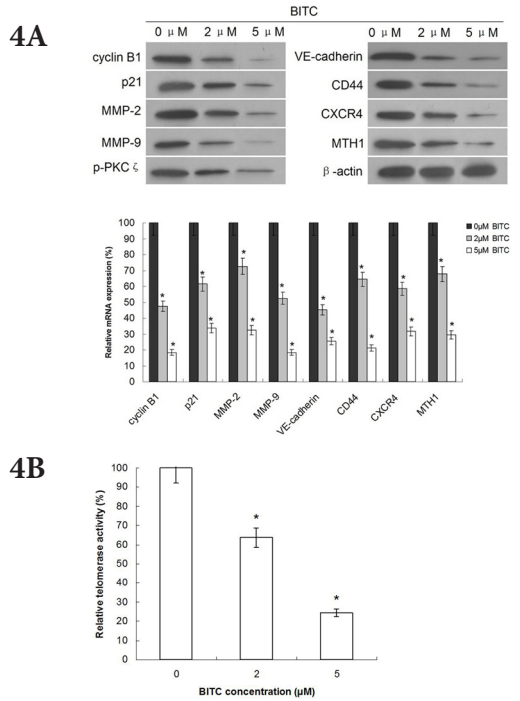

Figure 4. The Effect of BITC on Eexpresson of Tumor-Related Gene of Human Glioma U87MG Cells. (A) The effect of BITC on protein expression of cyclin B1, p21, MMP-2/9, VE-cadherin, CD44, CXCR4, MTH1 and *p*-PKCζ of U87MG cells was determined by Western blot assay. β-actin was used as an internal control for loading. **(B)** The effect of BITC on mRNA expression of cyclin B1, p21, MMP-2/9, VE-cadherin, CD44, CXCR4 and MTH1 of U87MG cells was determined by realtime PCR assay. GAPDH was used as an internal control for loading. Bars mean±SD. *p<0.05, compared with 0μM group, n=5. **(C)** The effect of BITC on activity of telomerase in U87MG cells was determined by ELIASA assay. Bars mean±SD. *p<0.05, compared with 0μM group, n=10

Table 2. Tumor-Related microRNAs Modulated by BITC

microRNA	Fold change	microRNA	Fold change
miR144	8.7	miR-21	-2.3
miR-122	6.5	miR-155	-2.6
miR-146a	5.6	miR-10a	-2.7
miR-335	4.8	miR-29b	-4.5
miR-34a	4.5	miR-373	-4.9
miR-126	3.7	miR-9	-5.8
miR-127	3.4	miR-181b	-6.6

of the telomerase and PKCζ by 2μM and 5μM BITC treatment for 24h. Further, BITC regulates expresson of tumor-related gene and microRNAs of human glioma U87MG cells by 2μM and 5μM BITC treatment for 24h (Table 1 and 2).

Discussion

Glioma is one of the most common primary malignant brain tumor in China and effective chemotherapy has become an alternative therapeutic due to the patients lost the opportunity for operation. In the 1990s, studies found that cruciferous vegetable was able to reduce the prevalence of cancer and isothiocyanate was activity compound in above plants (Pawlik et al., 2012; Singh et al., 2012). BITC is one of the isothiocyanates with the highest activity, however, the effect of BITC on glioma has not been full studied.

In this study, we first examine whether BITC was able to inhibit the adhesion and invasion of U87 MG, we found that BITC could inhibit the adhesion and invasion of U87 MG *in vitro*. Many types of tumors including glioma could develop a vascularization process via angiogenesis to provide an alternative tumor blood supply model such as vasculogenic mimicry (VM). Therefore, we also examined the effect of BITC on the vasculogenic mimicry of U87 MG, and we found that BITC was able to inhibit the vasculogenic mimicry by 3D culture.

To further understand the inhibition mechanism of invasion and angiogenesis, we study the cell cycle distribution of the tumor cells and the result showed that BITC significantly arrested tumor cell cycle at G2/M phase. We also study the effect of BITC on tumor-related gene of human glioma U87MG cells such as adhesion molecule, pro-angiogenesis gene, cell cycle regulation gene, matrix metalloproteinases, and we found that there was a decreased mRNA and protein expression of cyclin B1, p21, MMP-2/9, VE-cadherin, CD44 and CXCR4 and the activity of the telomerase and PKCζ which regulate the potential of invasion and angiogenesis by 2 μM and 5 μM BITC treatment for 24h (Hunakova et al., 2009; Lai et al., 2010; Kwiatkowska et al., 2013). And gene chip and microRNAs chip assay showed that BITC could up-regulate a series of expression of tumor-suppressive gene and microRNAs in U87MG cells, such as CRYAB, PTEN, miR144, miR-122 and so on, meanwhile, down-regulate oncogenic gene and microRNAs such as NUDT1, MAPK9, miR-181b, miR-9 and so on.

Especially, oxidative stress was one major anti-tumor mechanism for anti-tumor drug and we found BITC could enhance ROS production and suppress GSH activity in our prevenient study which was a major tumor antitoxicant molecule in tumor cells (Gayathri et al., 2009; Deeb et al., 2012; Kohsaka et al., 2013) We also found that BITC could suppress expression of MTH1 which is not essential for normal cells, however, it is essential for tumor cells by repairing DNA oxidative damage, and that suppression of MTH1 can significantly reduce the aggravation of tumor cells (Rai, 2012; Gad et al., 2014). Therefore, we considered that oxidative stress was a major mechanism of BITC on invasion and angiogenesis inhibition.

Acknowledgements

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