

# Benzyl isothiocyanate alters the gene expression with cell cycle regulation and cell death in human brain glioblastoma GBM 8401 cells

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**Abstract.** Glioblastoma multiforme (GBM) is a highly malignant devastating brain tumor in adults. Benzyl isothiocyanate (BITC) is one of the isothiocyanates that have been shown to induce human cancer cell apoptosis and cell cycle arrest. Herein, the effect of BITC on cell viability and apoptotic cell death and the genetic levels of human brain glioblastoma GBM 8401 cells *in vitro* were investigated. We found that BITC induced cell morphological changes, decreased cell viability and the induction of cell apoptosis in GBM 8401 cells was time-dependent. cDNA microarray was used to examine the effects of BITC on GBM 8401 cells and we found that numerous genes associated with cell death and cell cycle regulation in GBM 8401 cells were altered after BITC treatment. The results show that expression of 317 genes was upregulated, and two genes were associated with DNA damage, the DNA-damage-inducible transcript 3 (DDIT3) was increased 3.66-fold and the growth arrest and DNA-damage-inducible  $\alpha$  (GADD45A) was increased 2.34-fold. We also found that expression of 182 genes was

downregulated and two genes were associated with receptor for cell responses to stimuli, the EGF containing fibulin-like extracellular matrix protein 1 (EFEMP1) was inhibited 2.01-fold and the TNF receptor-associated protein 1 (TRAP1) was inhibited 2.08-fold. BITC inhibited seven mitochondria ribosomal genes, the mitochondria ribosomal protein; tumor protein D52 (MRPS28) was inhibited 2.06-fold, the mitochondria ribosomal protein S2 (MRPS2) decreased 2.07-fold, the mitochondria ribosomal protein L23 (MRPL23) decreased 2.08-fold, the mitochondria ribosomal protein S2 (MRPS2) decreased 2.07-fold, the mitochondria ribosomal protein S12 (MRPS12) decreased 2.08-fold, the mitochondria ribosomal protein L12 (MRPL12) decreased 2.25-fold and the mitochondria ribosomal protein S34 (MRPS34) was decreased 2.30-fold in GBM 8401 cells. These changes of gene expression can provide the effects of BITC on the genetic level and are potential biomarkers for glioblastoma therapy.

## Introduction

Glioblastomas are the most frequent and aggressive primary brain cancers in adults (1) with a high recurrence and mortality rate (2). Glioblastoma prognosis is poor and there are limited therapeutic options. In recent years, advances have been made in multimodality including surgery, radiotherapy, chemotherapy and biotherapy, but the overall 5-year survival rate is still <3% for patients with glioblastoma (3). Thus, we try to identify prognostic gene expression (upregulation or downregulation) that may contribute to evaluate a more effective treatment to improve patient survival and to address more precisely the use of comprehensive therapy.

Benzyl isothiocyanate (BITC), one of the isothiocyanates, is present in cruciferous plants, it acts against carcinogenesis (4,5) and induces cell death through the induction of apoptosis and cell cycle arrest in various human cancer cells (6-10). In human prostate cancer cells, BITC promoted the phosphorylation of Bcl-xL with simultaneous

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cell cycle arrest and subsequent apoptosis (11). In our previous studies we have demonstrated that BITC inhibited migration and invasion in human colon (12) and gastric (13) cancer cells *in vitro*. There is no available information to show whether BITC affects human brain tumor cells, in particular regarding the effects of BITC on gene expression in human glioblastoma cells.

In cell survival, to maintain the integrity of genomic and mitochondrial DNA is critically important. It was reported that damage to nuclear and mitochondrial DNA can increase the accumulation of defective cellular components leading to impact unfavorably on physiological functions, increasing entropy (14). If an agent induces DNA damage, the cell in order to respond to the DNA damage, activates the cell cycle checkpoints (G1, S and G2/M) to stop cell cycle progression in order to allow time for repair, thereby preventing transmission of damaged or incompletely replicated chromosomes (15). Thus, the associated gene expression regarding cell cycle progression, cell apoptosis and DNA damage in cells are important for cancer cell therapy. There is no previous study showing the anticancer properties of BITC at the genetic level of human glioblastoma. We investigated the effects of BITC on gene expression in human brain cancer glioblastoma multiforme (GBM 8401) *in vitro*.

## Materials and methods

**Chemicals and reagents.** BITC, dimethyl sulfoxide (DMSO), penicillin-streptomycin and trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA). RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from Gibco-BRL/Invitrogen (Carlsbad, CA, USA). Tissue culture flasks and plates were obtained from Gibco-BRL/Invitrogen.

**Cell culture.** Human brain glioblastoma GBM 8401 cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured following the supplier's instructions. Cells were grown in 75 cm<sup>2</sup> culture flasks with RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin and maintained in an atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. The medium was changed every 2 days (16).

**Cell morphological changes and viability assays.** GBM 8401 cells (8x10<sup>4</sup> cells/ml) were seeded into a 12-well plate containing RPMI-1640 medium for 24 h. In addition, BITC was added to wells at the final concentration of 6 µM for 0, 12, 24 and 48 h. After treatment, cells were examined and photographed using contrast-phase microscopy at a magnification of x400 and then harvested for measuring the total percentage of viable cells using flow cytometric assay (16).

**Annexin V/PI staining for cell apoptosis.** GBM 8401 cells (8x10<sup>4</sup> cells/ml) were seeded into a 12-well plate for 24 h and then treated with BITC (0 and 6 µM) for 0, 12, 24 and 48 h, and the cells were collected, washed with phosphate-buffered saline (PBS) and stained with Annexin V/propidium iodide (PI) staining kit (BD Biosciences, San Diego, CA, USA) (17). All samples were then immediately analyzed by flow cytometry.

**cDNA microarray assay for gene expression.** GBM 8401 cells (2.4x10<sup>6</sup> cells/dish) were maintained in a 10 cm dish for 24 h. Cells were treated with 6 µM BITC or DMSO for 48 h then cells were collected, and washed twice with PBS. All samples were further isolated in total RNA using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA). The isolated RNA was further conducted for cDNA synthesis, labeling and microarray hybridization. The fluorescent-labeled cDNA hybridization (Affymetrix GeneChip Human Gene 1.0 ST array; Affymetrix, Santa Clara, CA, USA) on the chip was conducted (18), and the fluorescence from each sample was measured by Asia BioInnovations Corporation (Taipei, Taiwan). Expression Console software (Affymetrix) with default RNA parameters (19,20) was used to analyze the data. BITC affecting gene expression with at least a 2-fold-change was considered significant and recorded.

## Results

**Cytotoxic effects of BITC in GBM 8401 cells.** To investigate the cytotoxic effects of BITC in GBM 8401 cells, after treatment of cells with 6 µM BITC for 0, 12, 24 and 48 h, the cell morphological changes and percentage of viable cells were measured and results are presented in Fig. 1A and B, respectively. BITC induced cell morphological changes and decreased cell viability in GBM 8401 cells and these effects are time-dependent (Fig. 1A and B).

**Induction of cell apoptosis in GBM 8401 cells after exposure to BITC.** In order to further examine whether cell death was induced by BITC and through the induction of cell apoptosis, the cells after treatment with 6 µM BITC were harvested and apoptotic cells were measured by Annexin V/PI staining, and the results are presented in Fig. 2. Based on the data in Fig. 2, BITC-induced apoptotic cell death and these effects are time-dependent. The treatment of cells with BITC increased the total apoptotic cell death to 36.81% at 48 h (Table I). The result is consistent with the morphology and examination of total viable cells.

**BITC alters the regulations of gene expression in GBM 8401 cells.** GBM 8401 cells were treated with or without 6 µM BITC for 48 h and then harvested for total RNA extraction. The expression of the top 10 up- and downregulated genes was estimated by cDNA microarray analysis and the results are presented in Tables II and III. BITC induced 317 upregulated genes and 182 downregulated genes of GBM 8401, respectively. Forty-six genes were upregulated in the range >3-<4-fold, and 198 genes were upregulated >2-<3-fold. One gene was downregulated >4-fold, and 11 genes were downregulated in the range >3-<4-fold, and 170 genes were downregulated >2-<3-fold (data not shown).

**Alterations in gene expression scored in GBM 8401 cells after exposure to BITC.** The data from GeneGo analysis were mapped and are shown as upward thermometers in red color and indicate upregulated signals and downward (blue) ones indicate downregulated expression levels of the genes as presented in Figs. 3-5. Fig. 3 shows the Development\_Hedgehog and PTH signaling pathways in bone and

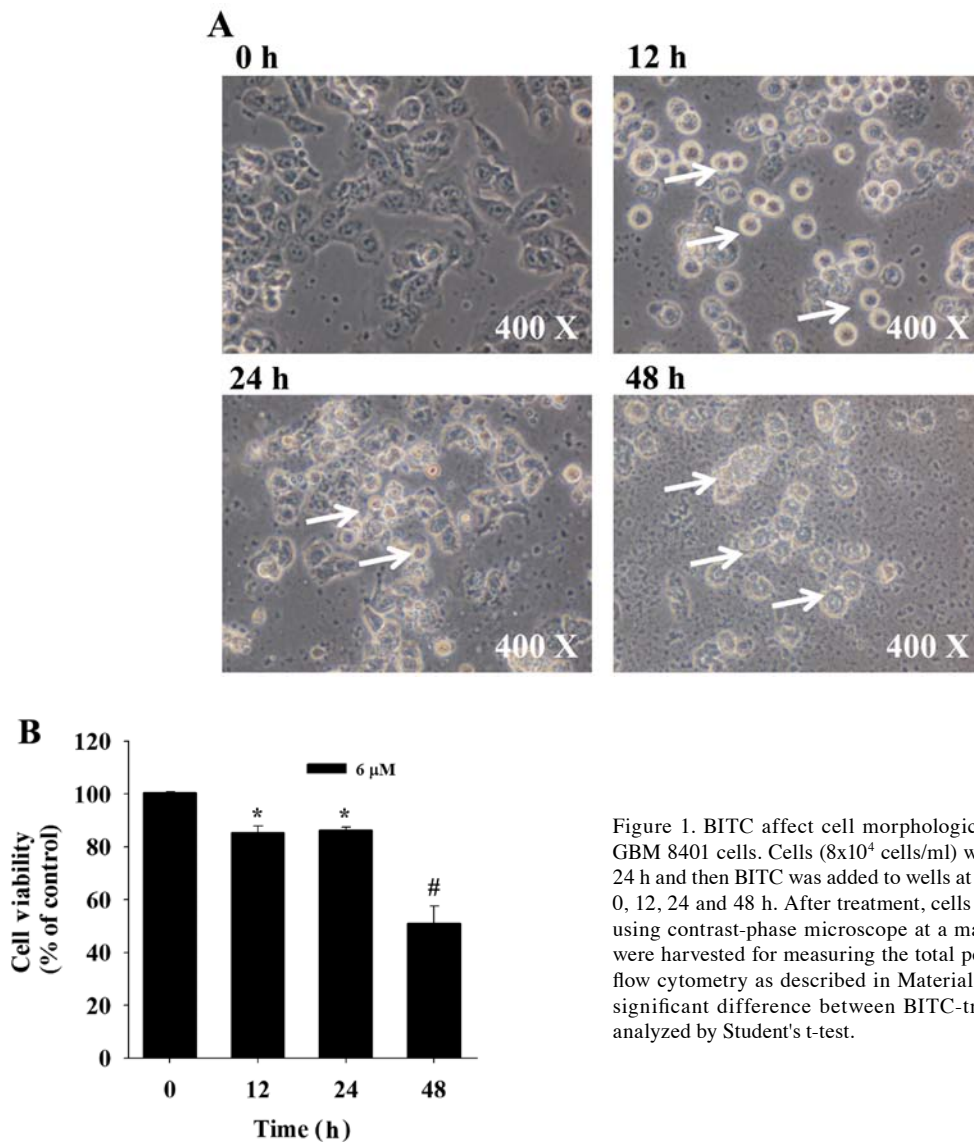


Figure 1. BITC affect cell morphological changes and total viability of GBM 8401 cells. Cells ( $8 \times 10^4$  cells/ml) were cultured in a 12-well plate for 24 h and then BITC was added to wells at the final concentration of  $6 \mu\text{M}$  for 0, 12, 24 and 48 h. After treatment, cells were examined and photographed using contrast-phase microscope at a magnification of  $\times 400$  (A) and then were harvested for measuring the total percentage of viable cells (B) using flow cytometry as described in Materials and methods. \* $p < 0.05$ , # $p < 0.001$  significant difference between BITC-treated groups and the control as analyzed by Student's t-test.

Table I. BITC-induced apoptosis of GBM 8401 cells.

Hours	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)
0	95.69 $\pm$ 1.24	1.87 $\pm$ 0.47	2.33 $\pm$ 0.77
12	70.34 $\pm$ 1.04 <sup>b</sup>	11.48 $\pm$ 1.90 <sup>b</sup>	15.51 $\pm$ 3.91 <sup>b</sup>
24	66.15 $\pm$ 3.44 <sup>b</sup>	24.62 $\pm$ 1.80 <sup>b</sup>	7.87 $\pm$ 1.41 <sup>a</sup>
48	59.35 $\pm$ 2.80 <sup>b</sup>	28.88 $\pm$ 1.32 <sup>b</sup>	7.93 $\pm$ 0.67 <sup>a</sup>

Cells were treated with  $6 \mu\text{M}$  of BITC for 0, 12, 24 and 48 h before the cells were stained using Annexin V/PI staining and analyzed by flow cytometry as described in Materials and methods. Data were obtained from three independent experiments. <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.001$  significant difference between BITC-treated groups and the control as analyzed by Student's t-test.

cartilage development. Fig. 4 shows the transcription and epigenetic regulation of gene expression and Fig. 5 shows the Development\_TGF- $\beta$ -dependent induction of EMT via MAPK.

## Discussion

Numerous studies have shown that BITC present biological activities including anticancer function *in vitro*. In the present study, BITC-induced cell morphological changes (Fig. 1A) and decreased the percentage of viable GBM 8401 cells and these effects are time-dependent (Fig. 1B). We also used Annexin V/PI staining to show that BITC-induced cell death through the induction of cell apoptosis in GBM 8401 cells (Fig. 2 and Table I) these effects are time-dependent. In order to further examine whether or not BITC affects gene expression of GBM 8401 cells, we treated cells with  $6 \mu\text{M}$  of BITC for 24 h before cells were harvested, total RNA was extracted for cDNA microarray and underwent further analysis for gene expression and the results are shown in Tables II and III.

It is well documented that after cells are exposed to anticancer agents, it may cause DNA damage or induce cell cycle arrest for causing cell death (21-23). We found that BITC decreased total viable cell number (Fig. 1B) based on cells incubated with BITC and then harvesting and staining by PI and examination by flow cytometric assay as previ-

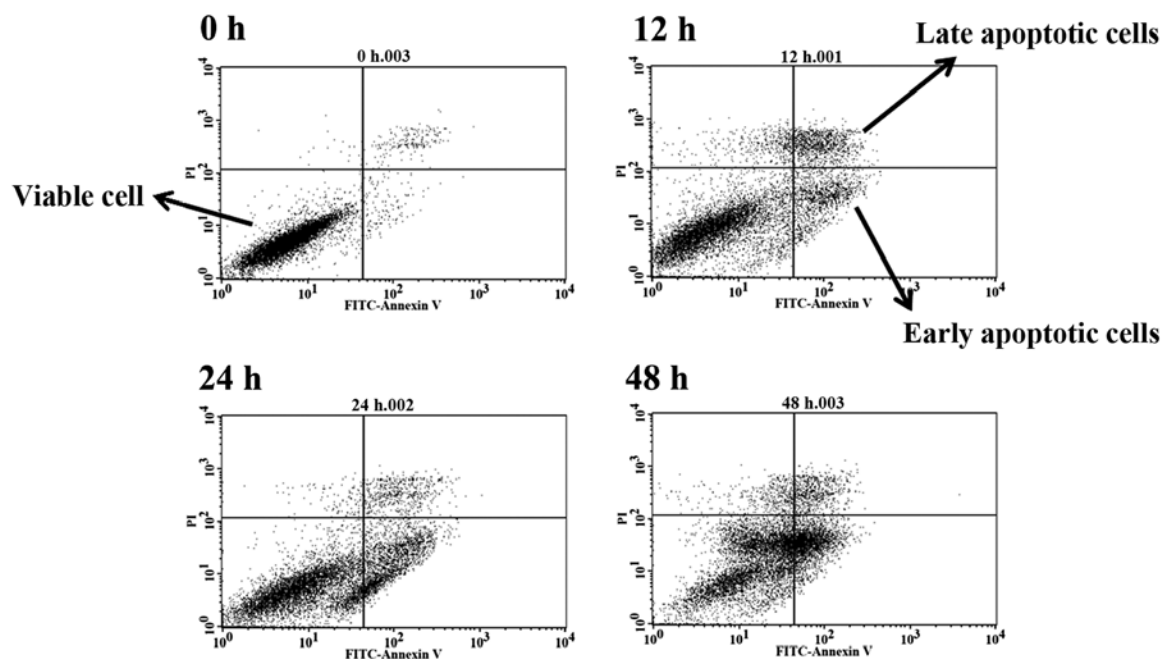


Figure 2. BITC induces apoptosis of GBM 8401 cells. Cells were treated with 6  $\mu$ M of BITC for 0, 12, 24 and 48 h before the cells were stained using Annexin V/PI staining and were analyzed by flow cytometry as described in Materials and methods. Data profiles of flow cytometric assay.

Table II. The top 10 upregulated genes of GBM 8401 cells by BITC treatment.

Fold-change	Gene symbol	mRNA description
11.04	<i>LPAR6</i>	Lysophosphatidic acid receptor 6
9.11	<i>LOC344887</i>	NmrA-like family domain containing 1 pseudogene
8.26	<i>EGR1</i>	Early growth response 1
7.65	<i>CLU</i>	Custerin
7.56	<i>CTH</i>	Cystathionase (cystathionine $\gamma$ -lyase)
6.05	<i>SATI</i>	Spermidine/spermine N1-acetyltransferase 1
5.81	<i>SLC7A11</i>	Solute carrier family 7 (anionic amino acid transporter light chain, xc-system), member 11
5.09	<i>AKR1B10</i>	Aldo-keto reductase family 1, member B10 (aldose reductase)
4.90	<i>HIST2H4B</i>	Histone cluster 2, H4b; H4a; histone cluster 4, H4; histone cluster 1, H4l; H4e; H4b; H4h; H4c; H4j; H4k; H4f; H4d; H4a; H4i
4.62	<i>SLCO1B7</i>	Solute carrier organic anion transporter family, member 1B7 (non-functional)

BITC, benzyl isothiocyanate.

ously described (24,25). We also confirmed cell apoptosis by Annexin V/PI staining and evaluation by flow cytometry and results indicated that BITC significantly induced cell death in GBM 8401 cells *in vitro* (Fig. 2).

Table II indicates that expression of 317 genes was promoted, and among them two genes associated with DNA damage in GBM 8401 cells, the DNA-damage-inducible transcript 3 (DDIT3) was increased 3.66-fold, and the growth arrest and DNA-damage-inducible  $\alpha$  (GADD45A) was increased 2.34-fold. Based on these observations, BITC induced DNA damage as shown previously (9), our results indicated that BITC-induced DNA damage was associated

with gene expression. Table II indicates that BITC also promoted four heat protein gene expression, the heat shock protein 70 kDa family member 13 (HSPA13), which was increased 2.16-fold, the heat shock protein 70 kDa protein 1A, 1B (HSPA1A) increased 2.13-fold [heat shock protein 90 kDa  $\beta$  (Grp94), membrane 2, pseudogene (HSP90B2P)] and increased 2.03-fold. It was reported that heat shock proteins (HSPs) have anti-apoptotic properties and they are often elevated in many human cancers; furthermore, the overexpression of HSPs is associated with poor survival and response to therapy (26-28). HSP expression in selected brain tumor cell lines (27,29) have been reported using

Table III. The top 10 genes of GBM 8401 cells downregulated by BITC treatment.

Fold-change	Gene symbol	mRNA description
-3.11	<i>LOC730755</i>	Keratin associated protein 2-4-like; 2-1; 2-4
-3.12	<i>DKC1</i>	Dyskeratosis congenita 1, dyskerin; small nucleolar RNA, H/ACA box 56
-3.18	<i>HIST1H1D</i>	Histone cluster 1, H1d
-3.24	<i>ACTC1</i>	Actin, $\alpha$ , cardiac muscle 1
-3.28	<i>EDN1</i>	Endothelin 1
-3.71	<i>HIST1H2AB</i>	Histone cluster 1, H2ab; histone cluster 1, H2ae
-3.74	<i>HIST1H2BI</i>	Histone cluster 1, H2bi; H2bc; H2be; H2bf; H2bg
-3.76	<i>TRNAU2</i>	Transfer RNA selenocysteine 2 (anticodon UCA)
-3.77	<i>KRT81</i>	Keratin 81
-4.43	<i>VTRNA1-1</i>	Vault RNA 1-1

BITC, benzyl isothiocyanate.

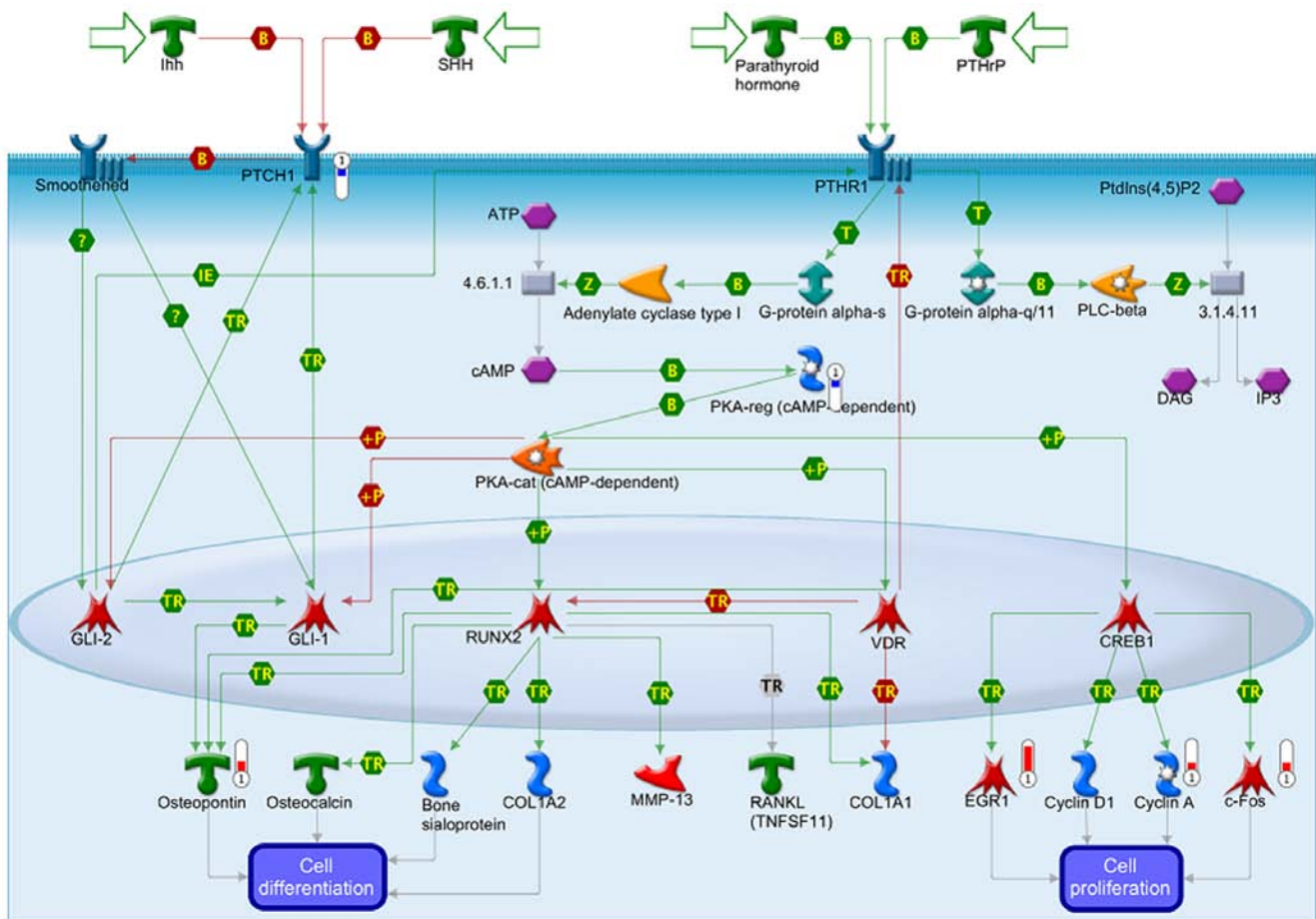


Figure 3. Development\_Hedgehog and PTH signaling pathways in bone and cartilage development. The top scored map (map with the lowest p-value) based on the enrichment distribution sorted by ‘Statistically significant Maps’ set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Upward thermometers have red color and indicate upregulated signals and downward (blue) ones indicate downregulated expression levels of the genes.

mainly immunohistochemistry (29-31). Table II indicates that BITC also promoted expression of seven genes associated with cell cycle such as CLK (CDC-like kinase 4), which was increased 3.29-fold, CCNG2 (cyclin G2) was increased 3.19-

fold, cyclin A1 (CCNA1) increased 2.30-fold, cyclin Y-like 1 (CCNYL1) increased 2.20-fold, cyclin-dependent kinase-like 5 (CDKL5) increased 2.19-fold, cyclin D binding myb-like transcription factor 1 (DMTF1) increased 2.04-fold and



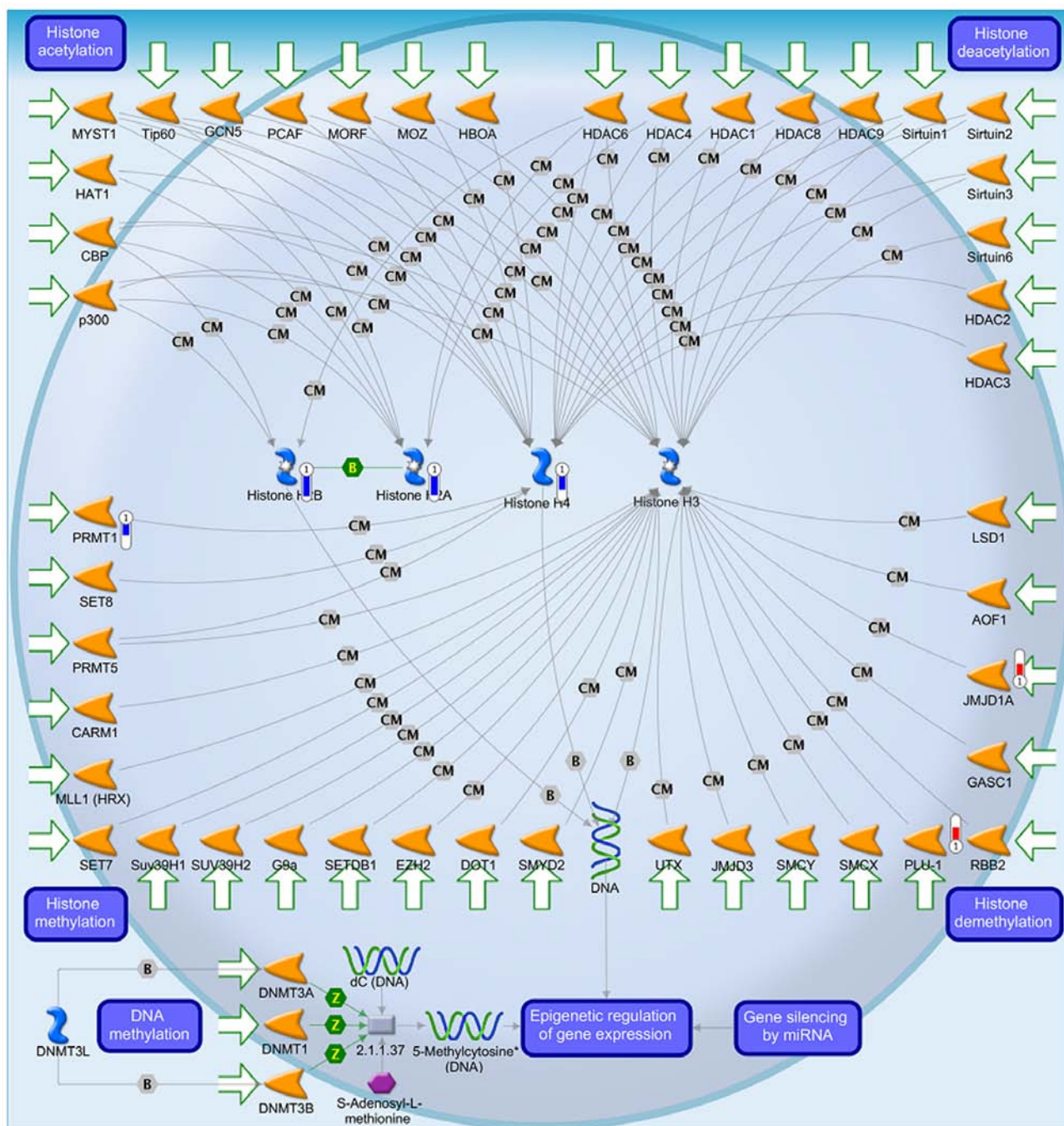


Figure 4. Transcription and epigenetic regulation of gene expression. The second scored map (map with the second lowest p-value) based on the enrichment distribution sorted by 'Statistically significant Maps' set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Upward thermometers have red color and indicate upregulated signals and downward (blue) ones indicate downregulated expression levels of the genes.

cell cycle progression 1 (CCPG1) was increased 2.01-fold in GBM 8401 cells.

Table III indicates that it suppressed expression of 182 genes in GBM 8401 cells, and among them a gene associated with receptor for cell responses to stimuli, the EGF containing fibulin-like extracellular matrix protein 1 (EFEMP1) was inhibited 2.01-fold, and the TNF receptor-associated protein 1 (TRAP1) was inhibited 2.08-fold. Both receptors are associated with cell sensitivity for stimuli agents (32,33). Furthermore, BITC inhibited mitochondria ribosomal genes such as mitochondrial ribosomal protein; tumor protein D52

(MRPS28) was inhibited 2.06-fold, mitochondria ribosomal protein S2 (MRPS2) decreased 2.07-fold, mitochondria ribosomal protein L23 (MRPL23) decreased 2.08-fold, mitochondria ribosomal protein S2 (MRPS2) decreased 2.07-fold, mitochondria ribosomal protein S12 (MRPS12) decreased 2.08-fold, mitochondria ribosomal protein L12 (MRPL12) decreased 2.25-fold and mitochondria ribosomal protein S34 (MRPS34) was decreased 2.30-fold in GBM 8401 cells. It is well documented that agents inducing cancer cell apoptosis are involved in the mitochondria (34,35), thus, in the present study, we found that BITC-induced cell death may be through

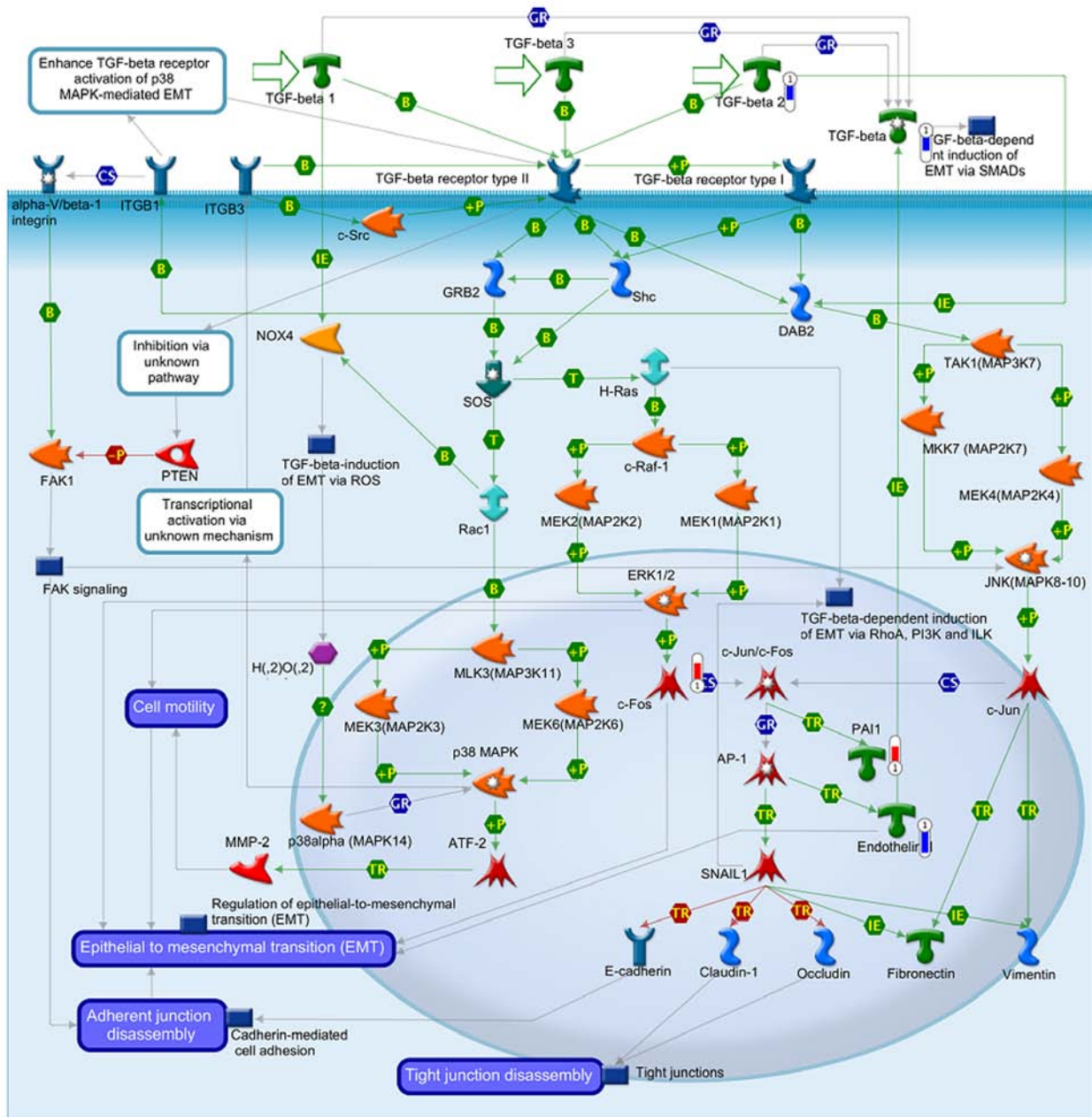


Figure 5. Development\_ TGF-β-dependent induction of EMT via MAPK. The third scored map (map with the third lowest p-value) based on the enrichment distribution sorted by ‘Statistically significant Maps’ set. Experimental data from all files is linked to and visualized on the maps as thermometer-like figures. Upward thermometers have red color and indicate upregulated signals and downward (blue) ones indicate downregulated expression levels of the genes.

the induction of DNA damage and affects mitochondria ribosomal gene expression in GBM 8401 cells.

In conclusion, we found that many genes are associated with DNA damage and cell cycle regulation and various genes that associated with the mitochondria were affected by BITC in GBM 8401 cells. These changes of gene expression in GBM 8401 cells, after exposure to BITC, provide further knowledge on the effects of BITC at the genetic level, and for future development of potential biomarkers for glioblastoma therapy.

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