Effect of overexpression of *HOX* genes on its invasive tendency in cerebral glioma

YUN-BAO GUO[1], YI-MENG SHAO[2], JING CHEN[3], SONG-BAI XU[1], XING-DONG ZHANG[4], MAO-REN WANG[5] and HAI-YAN LIU[4]

[1]Department of Neurosurgery, The First Hospital of Jilin University, Changchun, Jilin 130021; [2]Department of Surgery, Central Hospital of Changchun, Changchun, Jilin 130011; [3]Department of Orthopedics, China-Japan Union Hospital of Jilin University, Changchun, Jilin 130033; [4]Department of Anatomy, College of Basic Medical Sciences, Jilin University; [5]Department of Clinical Medicine, College of Jilin University, Changchun, Jilin 130021, P.R. China

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Abstract. Transcription factors encoded by *HOX* genes are vital in the determination of cell fate and identity during embryonic development. In certain malignancies, *HOX* genes also behave as oncogenes. The present study demonstrated suppression of the invasive tendency of glioblastoma multiforme U-118 and U-138 cells by the introduction of the antisense fragments of *HOXA6* and *B13* genes using electroporation. The invasion index indicated 79 and 72% reductions in the invasive ability of antisense *HOXA6* and *B13*, respectively. No significant differences in the invasive index of the parental and mock cells of each *HOX* gene were observed (invasive index, 0.75-0.91; P=0.05). A reduction in invasion tendency was also observed following betulinic acid (BA) treatment: The results from the matrigel assay analysis clearly demonstrated a significant inhibition in the invasive behaviour of U-118 and U-138 cell lines from day 15 following BA treatment, with a maximum effect on day 30. The invasion index demonstrated 62 and 65% reductions in invasion ability in the U-118 and U-138 cell lines, respectively. The suppression of *HOXC6* and *B13* expression by the introduction of the corresponding antisense fragments in addition to BA reduced invasion tendency in U-118 and U-138 cell lines. The mechanism underlying the association between the *HOX* gene and invasive behavior in glioma cells is yet to be understood. However, the anti-invasive behavior of BA may aid understanding of the mechanism in future studies.

Correspondence to: Professor Hai-Yan Liu, Department of Anatomy, College of Basic Medical Sciences, Jilin University, 126 Xinmin Street, Changchun, Jilin 130021, P.R. China
E-mail: haiyan@jlu.edu.cn

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Introduction

Glioblastoma multiforme (GBM), one of the most common malignant primary brain tumors, accounts for ~80% of brain tumors in adults and is the most fatal (1). The enhanced rate of proliferation and invasive growth are considered to be the characteristic features that result in the high morbidity and mortality of these tumors. The outcome of treatment for malignant gliomas is poor, with an average survival time of 10 months following therapy (2). Resistance to apoptosis, genomic instability and poor response to therapy are characteristics that exist in GBM (3). The current methods of treatment for glioma, including radiotherapy, surgery and chemotherapy (4,5), exhibit poor efficacy. Therefore, an improved understanding of the mechanisms underlying the initiation, progression and invasion of gliomas at the molecular and cellular levels are required for the development of novel therapeutic strategies.

A number of homeobox (HB) genes, including *SIX*, *MSX*, *PAX*, *LIM* and *HOX*, encode HB proteins (6). The transcription factors are encoded by *HOX* genes. There are 39 *HOX* genes in humans, whose function are partially or fully known (7). Out of two broad categories of HB genes, clustered HB (*HOX*) genes are involved in antero-posterior patterning during embryogenesis, while divergent HB (non-*HOX*) genes are dispersed throughout the genome (8). The 39 *HOX* genes in humans are arranged in clusters on four different chromosomes (*HOXA* at 7p15.3, *HOXB* at 17p21.3, *HOXC* at 12q13.3, and *HOXD* at 2q31) (9).

Observable differences in *HOX* gene expression exist in primary solid tumors, including those of the kidney (10), bladder (11), colon (12), lung (13), breast (14), prostate (15) and cervix (16). Hematopoietic malignancies involve the translocation of certain *HOX* genes. The enhanced expression of *HOXB3*, *HOXB4* and *HOXC6* (17,18) has been observed in childhood medulloblastomas and primitive neuroectodermal tumors (19,20).

Previously, it was reported that *HOXD* genes were expressed in neoplastic astrocytes (21) and pediatric low-grade gliomas (22). However, little is known about the function of

HOXD genes, particularly in gliomas. The present study analyzed the effect of the introduction of antisense *HOXC6*, *HOXB13* and betulinic acid (BA) on invasive behavior in human gliomas.

BA is a pentacyclic acid that has been reported to be effective on a number of types of human cancer (Fig. 1), including medulloblastoma, glioblastoma, Ewing's sarcoma (23), neuroblastoma (24) and leukemia (25). BA has been demonstrated to be effective on drug-resistant and -sensitive tumor cell lines, indicating its significant therapeutic benefit to chemotherapy-resistant patients with cancer (26). In addition, the impact of BA on hedgehog signalling was determined, which has been implicated in rhabdomyosarcoma development (27).

The present study aimed to investigate the effect of suppressing HOXC6 and B13 expression by introducing a corresponding antisense fragment on the invasion tendency of the GBM cell lines, U118 and U138.

Materials and methods

Cell lines and culture. The expression of all 39 *HOX* genes was examined in two human GBM cell lines (U-118 and U-138; American Type Culture Association, Rockville, MD, USA). The differentially expressed *HOX* (*A6, A7, A9, A13, D4*, and *D10*) genes were further examined in U87 and U373 GBM cell lines. Normal human astrocytes (NHAs; American Type Culture Association) were used as controls of non-neoplastic astrocytes. Cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS) and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air. Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Tissues. A total of 9 temporal lobes (TLs), 4 diffuse-type astrocytomas (WHO grades II, III, and IV) along with 28 human brain tissues were obtained by the surgical resection of patients admitted to The First Hospital of Jilin University (Changchun, China). Patients with hippocampal sclerosis and epilepsy served as the source of TL specimens. The patients under study varied in age between 34-75 years. Identification of the glioblastomas revealed that only one of the tumors was a secondary tumor. After extraction, the tissues were frozen immediately under liquid nitrogen and stored at -78˚C until further analysis. The study was approved by the local ethics committee at the University and all patients gave written consent.

Reverse transcription-polymerase chain reaction (RT-PCR). Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for the isolation of total RNA from the cells according to the manufacturer's instructions. The RNA samples were reverse transcribed into cDNA using synthesis kit (Invitrogen). The PCR amplification of the synthesized cDNA was carried out at 35 cycles in the mixture of 10 mM Tris-HCl (pH 8.3), 1.5 M $MgCl^2$, 50 mM KCl, 0.01% (w/v) gelatin, 200 nM dNTP, HOX-specific primers (0.5 nM each), and 2.0 U of platinum TaqDNA polymerase (Invitrogen). Every reaction involved two negative controls with deletion of the RT step or of the target cDNA.

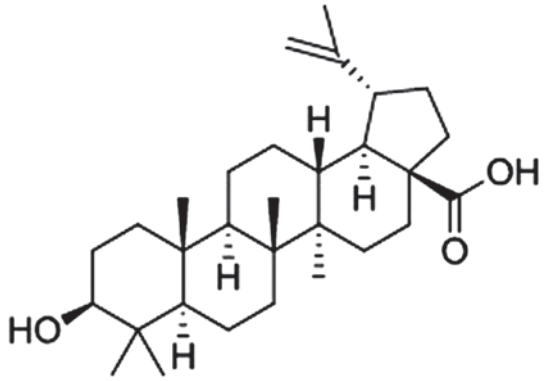

Figure 1. Structure of betulinic acid.

Antisense gene introduction of overexpressed HOX genes by electroporation. The NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov/nucleotide) was used to design the antisense fragments of HOXC6, B13 and C5 as the control. PCR was performed to generate antisense fragments of specific gene primers followed by introduction into a pGEM-Teasy™ vector (Invitrogen). The following primers were used: Forward, 5'-AGCAGCAGTACAAACCTGAG-3' and reverse, 5'-AGTGGAATTCCTTCTAAGC-3' for HOXA6; forward, 5'-TCAATCGCTCAGGATTTTAG-3' and reverse, 5'-AATTCCTTCTCCAGTTCCAG-3' for HOXC6; forward, 5'-ATGTGTTGCCAAGGTGAAC-3' and reverse, 5'-AACTTGTTGGCTGCATATC-3' for HOXB13; forward, 5'-CCCAGAGTCAATAAATAGTTGGACAA-3' and reverse, 5'-CCATAGTCCCTGCCACGAAT-3' for HOXC5. The amplifications were performed in 50-μl reaction volumes with an initial denaturation step at 94˚C for 5 min, prior to 38 thermal cycles of 94˚C for 1 min, 55˚C for 30 sec and 72˚C for 1 min, with a final extension at 72˚C for 10 min. To confirm the introduction of the PCR sequences into the vector a DNA sequencer (SEQ4X4 personal DNA sequencer; GE Healthcare, Little Chalfont, UK) was employed. *Eco*RI was used at 2 μM for the degradation of the vector and fragments formed were put into another vector pcDNA3.1 (Invitrogen Life Technologies), supplemented with Neomycin-resistance gene. *Apa*I was used at 2 μM for linearization of the vector (50 ng) containing each antisense fragment followed by electroporation with $2.6x10^6$ U-118 and U-138 cells (Gene Pulser, Bio-Rad). Following electroporation, the cells were cultured for 24 h and then used for G418 (neomycin) selection of the antisense introduced cells. The cells were cultured with G418 at 10 μM for 6 days followed by RT-PCR induced isolation of total RNA, using the Advantage™ RT-for-PCR kit (Biosciences Clontech, Inc., CA, USA). A total of 3 cell lines were established independently by introducing antisense of HOXA6, HOXB13 and HOXC5. Mock U-118 and U-138 cell lines were established as well.

Matrigel assay for the assessment of invasion of the cancer cells. Matrigel assay was used to study the invasion capabilities of 3 cell types, parental U-118 cell, mock U-118 (no antisense fragment) and antisense-introduced U-118. On the matrigel chamber membrane (Paragon Medical, Princeton, NJ, USA)

Table I. Percent of invasion and invasion index by antisense-introduced cells.

Cell line	2 h	4 h	8 h	16 h
HOXA6 (% in :I.I.)				
U-118	47	159	475	510
Mock cell	45.5:0.7	129.8:0.6	230.1:0.2	360.9:0.1
A6-L1	10.2:0.3	13.5:0.2	39.2:0.3	65.1:0.6
A6-L2	12.3:0.1	13.1:0.3	41.4:0.2	62.4:0.0
A6-L2	13.5:0.3	19.9:0.8	38.9:0.0	71.1:0.0
HOXB13 (% in :I.I.)				
U-138	38.2	161.1	290.5	370
Mock cell	37.8:0.5	121.7:0.3	218.1:0.6	310.6:0.6
B13-L1	17.5:1.0	24.2:0.1	61.3:0.0	110.2:0.0
B13-L2	17.8:0.1	59:3.4	89.7:0.2	112.1:0.2
B13-L3	17.0:3.3	50.7:0.1	82.9:0.2	122.8:0.3

Ratios are presented as % invasion of antisense gene introduced cells (L1, L2, L3 or mock): % invasion of parental cells.

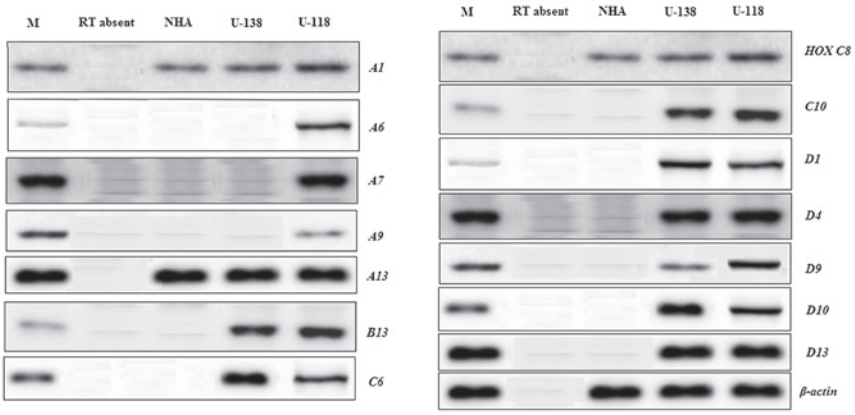

Figure 2. RT-polymerase chain reaction analysis of differentially expressed *HOX* genes in NHA and U-118 and U-138 glioblastoma multiforme cell lines. Control amplifications of β-actin are also shown. M, normal tissue (negative control without target cDNA); RT, reverse-transcription; NHA, normal human astrocytes.

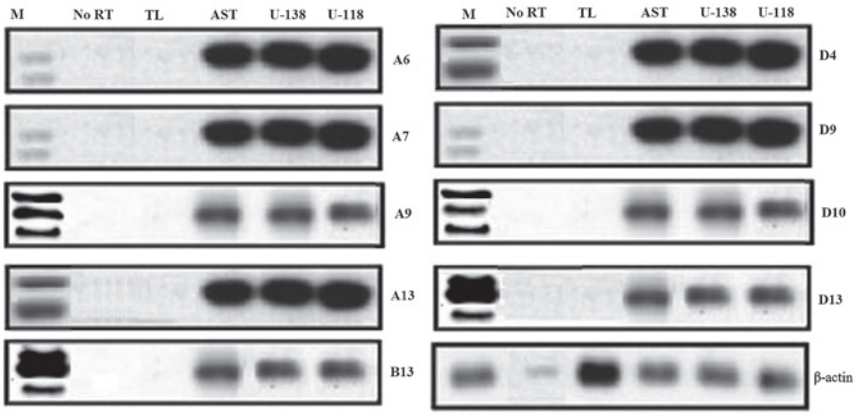

Figure 3. RT-polymerase chain reaction analyses demonstrating differentially expressed *HOX* genes in normal human TLs, AST samples (grades IV), and glioblastoma multiforme cell lines U-138 and U-118. M, normal tissue (negative control without target cDNA); RT, reverse-transcription; TL, temporal lobe; AST, astrocytoma.

$5x10^4$ cells were cultured for 20 h following the addition of 5 μM fibronectin. The cells passing through the matrigel were counted using 0.1% trypan blue stain (Sigma-Aldrich) on a Olympus IX-71 microscope (Olympus Corporation, Tokyo,

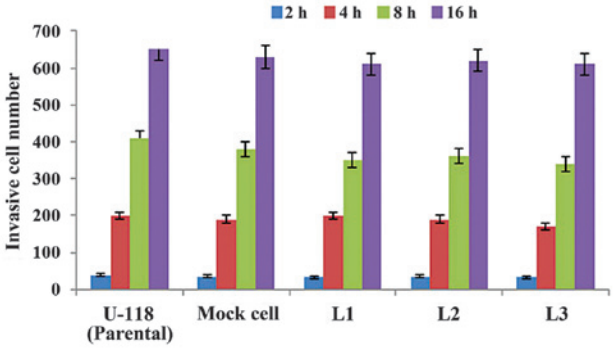

Figure 4. HOXC5 antisense-introduced U-118 cells were used as the control experiment. The chemoattractant used for the experiment was fibronectin. In this study, three independent cell lines, L1, L2 and L3, were used. The cells were put in the Matrigel™ chamber followed by culturing for 2-16 h. The number of cells penetrating the Matrigel™ are shown.

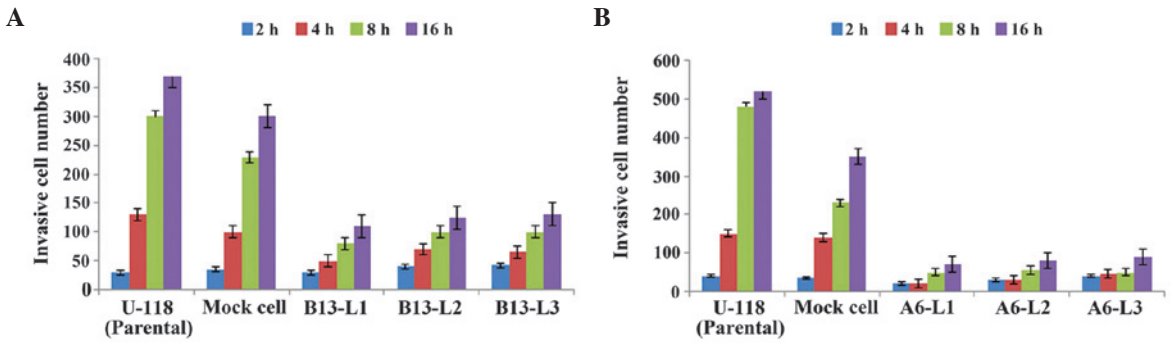

Figure 5. Reduction in invasive tendency of HOXB13 and HOXA6 antisense-introduced U-118 cells. (A) HOXB13 and (B) HOXA6. The difference in cell invasion of mock cells was non-significant; however, there was a significant reduction in invasive cells in antisense HOXB13 and HOXA6-introduced cells (P<0.05).

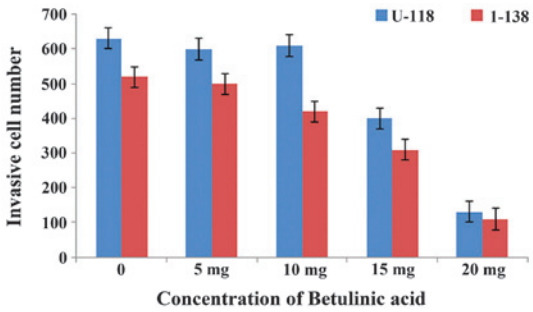

Figure 6. Matrigel assay analysis showing the decrease in the invasive behaviour of U-118 and U-138 cell lines with the increase in concentration of betulinic acid.

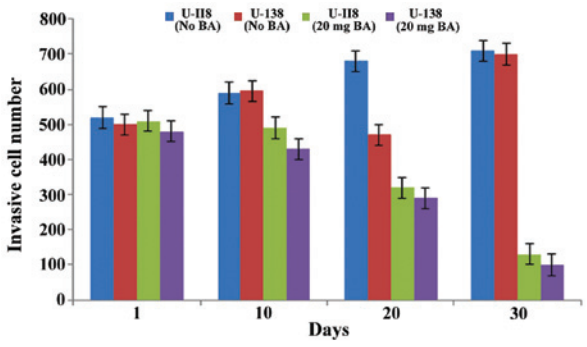

Figure 7. Effect of BA (20 mg) on invasive behavior in U-118 and U-138 cell lines over time. There was a significant reduction in invasive behavior in U-118 and U-138 cells treated with BA. BA, betulinic acid.

Japan). The percentage of invasion was calculated using the following formula:

$$\text{Invasion Index (II)} = \frac{\%\text{ invasion of antisense gene-introduced cells (L1, L2 or L3)}}{\%\text{ invasion of parental cells}}$$

Statistical analysis. Statistically significant values were determined using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD) and were evaluated with the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

HOX gene expression in normal human astrocytes and GBM cell lines. mRNA was extracted and cDNA was produced from U-118 and U-138 GBM cell lines and normal human astrocytes (NHAs) and gene expression was assessed using RT-PCR analysis. The results revealed expression of 26/39 *HOX* genes in NHAs in agreement with a previous study (28). *HOX A6, A7, A9, A13, B13, C6, C8, C10, D1, D4, D9, D10*, and *D13* were all silent in the NHA cell line (Fig. 2). There was an absence of such PCR products in cell cluster mRNA without reverse transcriptase which was used as the negative control. As such, no contamination was evident in the experiments.

HOX gene expression in normal TLs and astrocytomas from patient specimens. In TLs and patient astrocytomas, 9/39 HOX genes (*HOX A6, A7, A9, A13, B13, D4, D9, D10*, and *D13)* were not expressed in TLs and highly expressed in patient astrocytoma samples, as assessed by RT-PCR. The same 9 genes that were expressed in the patient astrocytoma samples were highly expressed in U-118 and U-138 cell lines (Fig. 3).

Chemoinvasion assay of U-118 and U-138 cells with HOXC6 and B13 antisense introduced. Among 9 HOX genes silent in NHAs and overexpressed in U-118 and U-138 cell lines, antisense fragments of HOX*A6* and *B13* were selected to analyze the invasive behavior of antisense introduced U-118 and U-138 cells. The 3 independent cell lines, including L1, L2 and L3 were established by successful introduction of HOXA6 and B13 antisense fragments using electroporation. In addition the antisense fragments of HOX*C5* (no overexpression) were also introduced in U-118 cells as a normal control. The antisense introduced and mock cells (vector only) served in the matrigel assay with fibronectin as a chemoattractant. In HOX*A6*- and B*13*-antisense-introduced cells, suppression of invasiveness in all three cell lines was observed; whereas, in the parental U-118 and mock cells of HOX*A6* and B*13* significant invasive behavior was observed. HOXC*5* demonstrated the expected degree of invasiveness (Fig. 4). Invasion index indicated 0.79 (79%), 0.72 (72%) reduction in invasion ability in HOX*A6*-, and B13-antisense-introduced cells respectively (Fig. 5), whereas no difference in the invasion index (0.75-0.91) between the parental and corresponding mock cells of each HOX gene was observed (Table I).

BA inhibits invasive behaviour of U-118 and U-138 cells. The invasive behaviour of U-118 and U-138 cell lines was also inhibited following treatment with BA. U-118 and U-138 cell lines were treated with a range of BA concentrations between 5-20 mg. There was a significant reduction in invasive behaviour at a concentration of 20 mg (Fig. 6). The results from RT-PCR analysis clearly demonstrated a significant inhibition in invasive behaviour of U-118 and U-138 cell lines from day 15 with a maximum effect on day 30. The invasion index demonstrated 0.62 (62%) and 0.65 (65%) reduction of invasion ability in U-118 and U-138 cells, respectively. However, no difference in the invasion index was observed between the parental U-118 and U-138 cell lines and mock cells of each (Fig. 7).

Discussion

Abnormal expression of HOX genes has been reported during a number of processes, including cell-cycle maintenance, proliferation of cells, angiogenesis, and penetration of cells to distant organs in numerous diseases (29-39). Therefore studying the role of overexpression of HOX genes on invasive tendency in cerebral glioma in the present study is merited. The effect of BA treatment on invasive tendency in U-118 and U-138 glioma cell lines was also studied.

The results from RT-PCR analysis revealed expression of 26/39 HOX genes in NHAs in agreement with an earlier study (28). HOX A6, A7, A9, A13, B13, C6, C8, C10, D1, D4, D9, D10, and D13 were all silent in the NHA cell line. RT-PCR analysis identified 9/39 HOX genes (HOX A6, A7, A9, A13, B13, D4, D9, D10, and D13) were highly expressed in patient astocytomas, which were not expressed in normal TL samples. The same 9 genes were highly expressed in U-118 and U-138 cell lines. Among these 9 HOX genes silent in NHAs and overexpressed in U-118 and U-138 cell lines, antisense fragments of HOXA6 and B13 were introduced into U-118 and U-138 cells to analyse the invasive behavior of the cells following reduction in expression of these HOX genes. In addition, the antisense fragments of HOXC5 (no overexpression) were also introduced in U-118 and U-138 cells as normal control. A marked reduction in invasive tendency was observed in all 3 cell lines in which HOXA6- and B13-antisense were introduced; whereas, parental U-118 and mock cells of HOXA6 and B13 did not demonstrate significant invasive behavior. It was observed that the degree of invasiveness by HOXC5 was within the expected limits. The invasion index indicated 0.79 (79%), 0.72 (72%) reduction of invasion ability in HOX-antisense-introduced U-118 and U-138 cell lines, respectively. By contrast, the invasion index for the parental cells when compared with mock cells for HOX genes demonstrated no significant changes.

Treatment of U-118 and U-138 cell lines with a range of BA concentrations from 5-30 mg resulted in a significant reduction in invasive behaviour at a concentration of 20 mg. The results from RT-PCR analysis clearly demonstrated a significant inhibition in invasive behaviour of U-118 and U-138 cell lines from day 15 with a maximum effect on day 30. The invasion index demonstrated a 0.62(62%) and 0.65 (65%) reduction of invasion ability in U-118 and U-138 cells, respectively. However no difference in invasion index was observed between the parental U-118 and U-138 cell lines and corresponding mock cells.

The present study suppressed HOXC6 and B13 expression by introducing corresponding antisense fragments, resulting in the reduction of the invasion tendency in U-118 and U-138 cell lines. It was also demonstrated that treatment with BA suppresses the invasion capabilities in U-118 and U-138 cell lines. It is yet to be fully understood how the enhanced expression of HOX genes is involved in controlling the invasive behaviour in glioma cells. However the anti-invasive behaviour of BA may aid in understanding the underlying mechanism in future studies.

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