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Chemotherapeutic Drug Resistant Cancer Stem-like Cells of Glioma

Mi Kyung Kang and Soo Kyung Kang*

Department of Physiology, Collgeg of Medicine, Pusan National University, Ami-Dong, Se-Gu, Busan, 602-739, Korea

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Glioblastoma multiforme (GBM) is the most frequently occurring brain cancer. Although the existence of cancer stem cells (CSCs) in GBM has been established, there is little evidence to explain the link between CSCs and chemoresistance. In this study, we investigated that only a few cells of A172 and established GBM2 survived after 1,3-bis(2-chloroethyl)-1-nitrosourea (BiCNU) exposures and these survived cells resist the subsequent BiCNU treatment. In addition, these BiCNU-resistant small populations derived from GBM cells increased the phosphorylations of Erk and Akt and highly expressed CD133 stem cell surface marker. Furthermore, we observed that the BiCNU-resistant cancer cells derived from GBM have grown tumors when transplanted into severe combined immuno-deficient (SCID) mouse brain. These results demonstrate that BiCNU-resistant subpopulation cells derived from GBM have cancer stem-like cell properties. Therefore, it may provide provide further evidence that CSCs in GBM have chemotherapeutic drug resistance.

Key words – Glioblastoma multiforme, cancer stem cells, chemoresistance, 1,3-bis(2-chloroethyl)-1-nitrosourea, tumor

Introduction

Glioblastoma multiforme (GBM) is the most frequent brain tumor. Compared with other tumors, GBM is relatively aggressive variant in human [23]. Besides surgery, which is an important initial therapeutic measure in this malignant glioma, postoperative radiotherapy and chemotherapy have been effective in GBM [13]. Despite constant efforts for the prevention and treatment of GBM, the GBM contained subpopulations of cells with resistance to conventional therapies that can repopulate the tumor after treatment [22]. Therefore, the identification of the cell types involved in resistant phenomena is critical to both scientific and therapeutic standpoint of GBM.

Chloroethylnitorsourea, especially BiCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), has been the most commonly used pharmacological agent in chemotherapy of GBM following surgical resection and radiation [5]. BiCNU passed across the blood-brain barrier and killed tumor cells via multiple cytotoxic actions including carbamoylation and alkylation of DNA [24]. But, GBM showed resistance to BiCNU chemotherapy leading to subsequent tumor growth. Several investigators have demonstrated that variation in multidrug resistance genes [12], DNA repair activity [19], and glutathione S-transferase and intracellular glutathione content [2] have been speculated to cause BiCNU chemoresistance in GBM.

Recently, it has been reported that cancers contain small cell populations with capability to sustain tumor formation and growth in tumor cells, termed cancer stem cells (CSCs) [10,17]. The CSCs have been identified in leukemia [3], multiple myeloma [14] and breast cancer [1,16]. Several studies reported that CSCs exist in human brain tumors [6,7,9,20] and CSCs can initiate tumors *in vivo* [21]. The CSCs shared many properties such as self-renewal and multi-potency with normal stem cells [10,17], and expressed a wide variety of transporters involved in drug efflux [4,25]. It seems that CSCs might have a drug resistant capacity and resulted in the recurrence of cancer after chemotherapy. However, there is little evidence to explain the link between CSCs and chemoresistance.

In this study, to investigate whether resistance of chemotherapy is associated with CSCs in GBM, we developed a dissociated cell system to facilitate the study of resistant cancer cells derived from A172 and GBM2 after BiCNU exposure. Also, we investigated whether BiCNU-resistant cancer cells possess cancer stem-like cell properties in culture and in severe combined immunodeficient (SCID) mice. These findings may provide evidence that CSCs in GBM have chemotherapeutic drug resistance. Also, it should allow the information for tumor cell biology and the ther-

*Corresponding author
Tel : +82-51-240-7961, Fax : +82-51-246-6001
E-mail : skkang@pusan.ac.kr

apeutic outcome of GBM.

Materials and Methods

Cell culture

Human glioblastoma multiforme cell line, A172, was obtained from American Type Culture Collection (Rockland, MD). Primary GBM tissue-derived cell line, GBM2, was established in the author's laboratory from a surgical specimen of 62-old-men as previously described [8]. A172 and GBM2 cells were cultured with DMEM containing 10% FBS and 1% penicillin-streptomycin. All the cells were maintained at 37°C in an atmosphere with 5% CO_2.

Determination of BiCNU sensitivity and isolation of BiCNU-resistant cells in human glioblastoma cell lines

In order to determine the drug sensitivity, A172 and established GBM2 cells ($1x10^4$ cells/well) were plated on 96-well plates and treated with various concentrations of BiCNU (0-132 μg/ml) and further incubated for 48 h. Cell viabilities were determined by trypan blue dye exclusion method. For isolation of drug resistant cells of the cultured cancer cells, A172 cells and established GBM2 cells were treated with 33 μg/ml of BiCNU (1,3-bis(2-chloroethyl)-1-nitrosourea, Sigma) and incubated at 37°C for 2 days. After exposure to BiCNU, the cells were rinsed 3 times with phosphate-buffered saline (PBS), fed with BiCNU free DMEM medium containing 10% FBS. After an additional 2- or 3-day incubation, the BiCNU-resistant cancer cells were divided and used at passage 2-3 for the experiment. Prior to and after BiCNU treatment, the number of cells was measured using the trypan blue dye exclusion method.

Immunoblot analysis of BiCNU-resistant glioblastoma cells

The BiCNU-resistant cancer cells derived from glioblastoma cell lines, A172 and GBM2, were lyses in 500 μl of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EGTA, 1 mM glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF). The cell lysates were centrifuged at 15,000 X g for 10 min, and then 30 μg of protein extracts were subjected to 10% SDS-PAGE analysis and transferred to the nitrocellulose membrane. The membrane was pre-incubated with PBS containing 5% skim milk, and probed with anti-pErk1/2 (1:1000, Cell Signaling, USA), anti-pAkt (1:1000, Cell Signaling) and anti-β-Actin (1;2000, Sigma) in PBS containing 5% skim milk for overnight at 4°C Following incubation with primary antibodies, the membranes were washed with PBS and subsequently incubated for 1 hr with IgG horseradish peroxidase conjugated secondary antibodies (1:1000, Cell Signaling). Enhanced chemo-luminescence (ECL, Amersham-Pharmacia, UK) system was used for detection. Relative band intensities were determined by quantitation of each band with Quantity-one-4,2,0 image analysis software (Bio-Rad, USA).

RNA isolation and reverse transcription-PCR Analysis of BiCNU-resistant glioblastoma cells

Total cellular RNA was extracted from the BiCNU-resistant cancer cells with Trizol (Invitrogen, USA). The cDNA was synthesized from 1 μg of total RNA with and oligo-dT primer (Promega, USA) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega) according to the menufacturer's instructions. The cDNA was amplified using 2 μl of the reverse transcriptase reaction products in 25 μl with 10 pmol of the specific primers for 35 cycles. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing and 1 min of extension at 72°C. All primer sequences were determined using established human GeneBank sequences for genes indicative of neural lineages or control genes (Table 1). Duplicate PCR reactions were amplified using primers designed GAPDH as an internal control for assessing PCR efficiency and for subsequent analysis by 1.5% agarose gel electrophoresis.

Table 1. List of primers used for the RT-PCR analysis

Gene	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)
Sox-2	TACCTCTTCCTCCCACTCCA	ACTCTCCTCTTTTGCACCCC
Nestin	ACCAAGAGACATTCAGACTCC	CCTCATCCTTATTTTCCACTCC
GFAP	GCTCGATCAACTCACCGCCAACA	GGGCAGCAGCGTCTGTCAGGTC
MAP2	CTTCTTGCAGCGATACAGCTC	ATGCTCCAATAAATTCACTGC
EGFR	CCTTTGGACACCTATTCAGG	GTGAGTGTGTCAGCTGGAAG
GAPDH	CATGACCACAGTCCATGCCATCACT	TGAGGTCCACCACCCTGTTGCTGTA

Flow-cytometric analysis of BiCNU-resistant glioblastoma cells

For analysis of subpopulations in the BiCNU-resistant cancer cells, the cells the cells were incubated at 4°C for 30 min with phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotec, Heidelberg, Germany). For an isotype control, PE-conjugated nonspecific mouse immunoglobulin G (IgG; Pharmingen) was substituted for the primary antibody. The labeled cells were analyzed and separated with FACS Vantage fluorescence-activated cell sorter (Beton Dickinson, CA), and then data were analyzed using CELLQuest dot ware (Beton Dickinson).

Cell transplantation of BiCNU-resistant glioblastoma cells into the SCID mice

For engraftment of BiCNU-resistant cancer cells, Athymic SCID mice were anesthetized with i.p. ketamine and radio-resistant A172 and GBM2 cells (5 X 10^5, respectively) were resuspended in 10 μl of HBSS after labeled with CFDA-SE cell tracer kit (Chemicon) before transplantation for confirmation of xenograft. The CFDA- labeled cells were injected into the right striatum (0.1 μl/ min) of 5-week-old SCID female mice (Jackson Laboratory, USA). The following coordinates were used: antero-posterior = 0 mm; mediolateral = 2.4 mm; dorso-ventral = 2.6 mm. The mice were sacrificed at 4 weeks post-transplantation. The mouse brains were immediately fixed with 4% paraformaldehyde and embedded in Tissue-Tek OCT (optimal culture temperature) compound (Miles, Elkhart, IN), and then frozen at -20°C. Cryostat sections (10 μm) were cut through the brain using a freezing microtome (CM3050; Leika Microsystems), mounted on poly-D-Lysine-coated slides (Sigma), and air-dried overnight at 37°C. The sections were processed as by Vescovi et al. [27]. The experiments were all conducted in accordance with the institutional guidelines established by the Pusan National University. In order to observe engrafted brain tissues, the tissue sections were stained with hematoxylin and eosin (H&E) and were examined and photographed with Phase contrast microscope (Nikon Microsystems).

Results

Brain cancer cells are resistant to chemotherapeutic drug

To assess the BiCNU sensitivity of GBM, A172 and established GMB2 cells were treated with various concentrations of BiCNU for 2 days and evaluated their viability using the trypan blue dye exclusion method. As shown in Figure 1, cell viabilities of GBM2 and A172 cells decreased in a BiCNU-dose dependent manner. Although A172 cells were more resist than GBM2 cells, most of BiCNU-treated cancer cells were died and only a few cells were survived after 2 days. In the case of 33 μg/ml BiCNU treatment, the survival rates of A172 and GBM2 cells were below 20.2% and 11.6%, respectively. These results indicated that GBM contains chemotherapeutic drug resistant cells.

In order to evaluate the BiCNU sensitivities of BiCNU-resistant cancer cells, we additionally treated with 33 μg/ml of BiCNU and isolated BiCNU-resistant cancer cells of A172 and GBM2 cells. Relative to cells grown without BiCNU, some the remaining cells exhibited smaller and rounder by BiCNU treatment (data not shown). These round-shaped small cells derived from A172 and GBM2 were more resistant to subsequent BiCNU treatment than parental GBM cells about 3.4- and 6.7-fold, respectively (Fig. 2). Moreover, in BiCNU-resistant cancer cells derived from A172 and GBM2, the phosphorylations of Erk1/2 and Akt were increased at 34% and 21%, (Fig. 3). These findings indicated that BiCNU-resistant cancer cells of GBM survived through the activation of Erk and Akt after BiCNU expose.

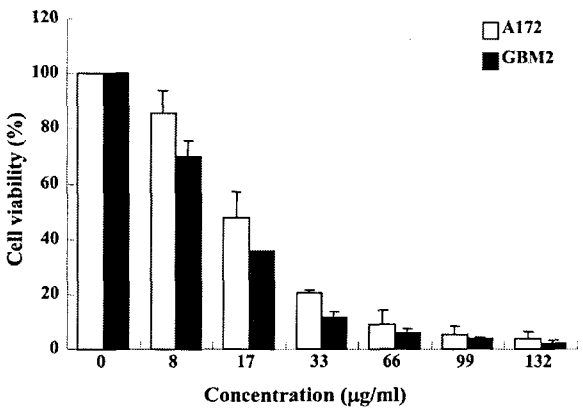

Fig. 1. Effects of BiCNU on the cell viability in human brain cancer cells. A172 and GBM2 cells were treated with various concentrations of BiCNU for 2 days. The cell viability was determined by trypan blue dye exclusion assay. □, A172 human glioblastoma cell line; ■, GBM2, primary culture from a human glioblastoma multiforme. Values are expressed as percentage of control, which was defined as 100%. Mean values for each treatment group were obtained from four to six samples obtained in three independent experiments. Data are plotted as the mean ± SD.

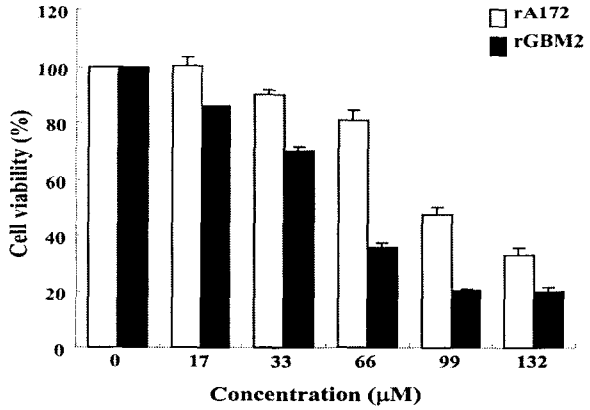

Fig. 2. Effects of BiCNU on the cell viability in BiCNU-resistant human brain cancer cells. BiCNU-resistant cancer cells derived from A172 and GBM2 cells were treated with various concentrations of BiCNU for 2 days. The cell viability was determined by trypan blue dye exclusion assay. □, A172 human glioblastoma cell line; ■, GBM2, primary culture from a human glioblastoma multiforme. Values are expressed as percentage of control, which was defined as 100%. Mean values for each treatment group were obtained from four to six samples obtained in three independent experiments. Data are plotted as the mean ± SD.

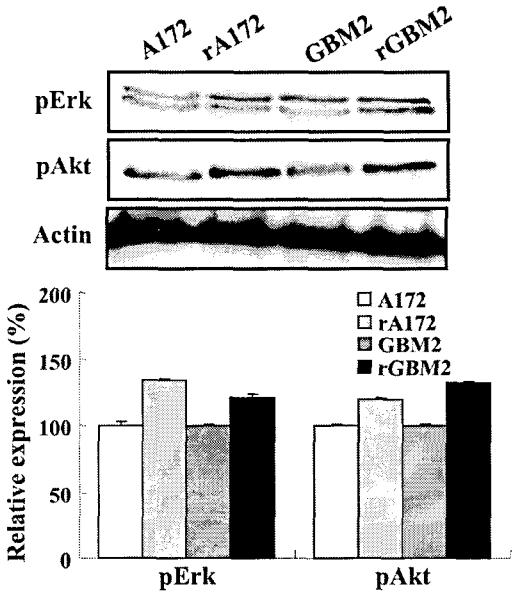

Fig. 3. Effects of BiCNU on the protein exptession in naive and BiCNU-resistant human brain cancer cells. The expression levels of Erk1/2 and Akt in cell lysates (30 μg) were determined by Western blotting with anti-phospho-Erk1/2 and -Akt. Expression of proteins was quantified by using the Quality-one-4,2,0 image analyzer as described in Materials and Methods. Values are expressed as percentage of control, which was defined as 100%. Results correspond to 3 independent experiments. Data are plotted as the mean ± SD.

Chemotherapeutic drug resistant cells in brain cancer cells contain a stem-like subpopulation

BiCNU-resistant cancer cells derived from GBM cell lines were stained with fluorescence-conjugated primary antibody against CD133 surface marker for stem cell, and were analyzed by flow cytometry. FACS analysis indicated that CD133 stem cell surface markers were highly expressed in BiCNU-resistant GBM cells (Fig. 4). BiCNU-resistant cancer cells derived from A172 and GBM2 cells contain 17.9 and 33.6% of CD133+ subpopulations, respectively. It was indicated that BiCNU-resistant GBM cells contained a stem-like cancer cells.

We performed RT-PCR analysis to determine whether BiCNU-resistant cancer cells derived from A172 and GBM2 were expressed multiple genes enriched in neural stem cells and cancer cells (Fig. 5). The mRNA expression patterns were differently expressed in two types of GBM cancer cells. Naïve A172 cells showed high expressions of Nestin, Sox-2 and EGFR. Conversely, Naïve GBM2 cells

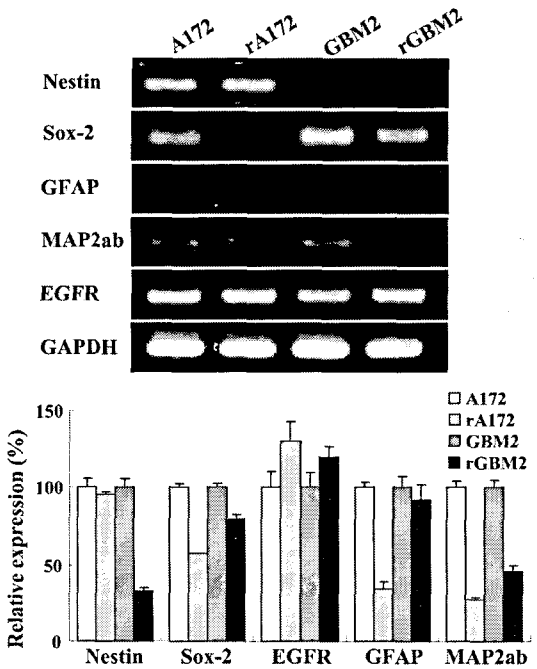

Fig. 4. Effects of BiCNU on the mRNA exptession in naive and BiCNU-resistant human brain cancer cells. The mRNA expression levels of multiple genes were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Expression of mRNAs was quantified by using the Quality-one-4,2,0 image analyzer as described in Materials and Methods. Values are expressed as percentage of control, which was defined as 100%. Results correspond to 3 independent experiments. Data are plotted as the mean ± SD.

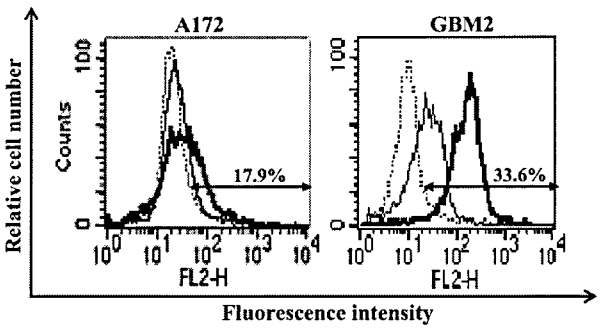

Fig. 5. Representative flow cytometic histograms illustrating the expression of CD133 surface epitope in parental and BiCNU-resistant human brain cancer cells. The cells were stained with CD133 coupled to PE (phycoerythrin) and analyzed by flow cytomery. -----, Isotype control; ——— , Immunophenotype of parental cells; ———, Immunophenotype of BiCNU-resistant cancer cells. Results correspond to 3 independent experiments.

highly expressed Sox-2 and EGFR and low expressed Nestin. These genes expression patterns were decreased in BiCNU-resistant GBM cells. On the other hand, EGFR mRNA expression pattern was slightly increased by BiCNU treatment in BiCNU-resistant cancer cells. It was supported that brain tumors exhibit phenotypic heterogeneity, being composed of cells expressing both undifferentiated and differentiated markers.

The tumorigenesis of chemotherapeutic drug resistant cancer stem-like cells derived from brain cancer cells

To determine whether BiCNU-resistant cancer cells derived from A172 and GBM2 cells were capable of tumor initiation *in vivo*, we transplanted these cells into a SCID mouse brain. BiCNU-resistant GBM cells could initiate tumors formation below the cell injection site after 4 weeks later (Fig. 6). As shown by hematoxylin and eosin staining, BiCNU-resistant GBM cells generated highly peculiar nest-like tumors contains cancerous tissue structure. The presence of regenerated tumor in transplanted SCID mice was suggested that the BiCNU-resistant cancer cells derived from A172 and GBM2 possess the strong capacity for tumorigenesis.

Discussion

A glioblastoma multiforme (GBM) is the most common and aggressive variant in human [23].

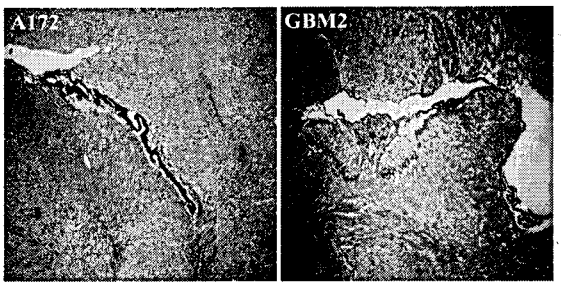

Fig. 6. *In vivo* tumorigeneisis of BiCNU-resistant cancer cells derived from human glioblastoma by transplanted subpopulation in an SCID mouse. Mice were transplanted with BiCNU-resistant A172 or GBM2 cells into the right striatum by stereotaxic injection and sacrificed at 4 wk post-transplantation. Frozen sections of brain tumors were stained with hematoxylin and eosin. Magnification, X100.

Besides surgery, which is an important initial therapeutic measure in this malignant glioma, postoperative radiotherapy and chemotherapy are considered the standard of treatment in these cases [13]. Among the chemotherapeutic drugs, BiCNU has been a mainstay in the adjunct chemotherapy of GBM [5,24]. However, BiCNU does not appear to substantially prolong median survival, even though the proportion of patients living more than 18 months increased from 5 to 15% [5]. Moreover, GBM contained subpopulations of cells with intrinsic resistance to chemotherapy that can repopulate the tumor after treatment [23].

In this study, we observed that only a few cells of A172 and GBM2 survived after BiCNU exposures (Fig. 1). In addition, these survived cells resist the subsequent BiCNU treatment (Fig. 2). These results indicated that brain tumors contain small cell populations with resistance to BiCNU chemotherapy [22]. While the BiCNU-sensitive cancer cells continued dying, the BiCNU-resistant cancer cells derived from A172 and GBM2 were survived and proliferated through the activation Erk and Akt after BiCNU expose (Fig. 3). This finding demonstrated that BiCNU-resistant cancer cells survive through the activation of Erk and Akt after BiCNU expose [26].

Previous report demonstrated that GBM tumors are organized in a hierarchy of heterogeneous cell populations with different biological properties [12,23]. Recently, several studies have identified CSCs in brain tumors [6,7,9], and CSCs have raised cancer growth and recurrence [20,21]. However, it is difficult to link chemotherapeutic resistance and CSCs of GBM. Moreover, it may not be pro-

vide the responsibility of recurrences in brain tumors after BiCNU chemotherapy.

Our FACS analysis results showed that BiCNU-resistant cancer cells derived from GBM contain a stem cells those expressing CD133 cell surface protein (Fig. 4). CD 133 cell surface marker was enriched in stem cells such as hematopoietic stem cells (HSCs), prostatic epithelial stem cells and endothelial precursors [11,15,18]. Also, this cell surface epitope is a marker of brain cancer stem cells [20,21]. Moreover, BiCNU-resistant cancer cells decreased the levels of mRNA expression on GFAP for astrocyte and MAP2ab for neurons and increased the levels of EGFR for cancer cells (Fig. 5). The BiCNU-resistant cancer cells can initiate tumor when transplanted into SCID mice (Fig. 6). These results indicated that BiCNU-resistant cancer cells derived from A172 and GBM2 cells are cancer stem-like cells with the abilities of proliferation and tumorigenesis. Therefore, BiCNU-resistnat cancer stem-like cells derived from A172 and GBM2 may account for their capability to escape conventional therapies, thus lead to disease relapse although the primary lesion is eradicated [6,21].

In conclusion, we investigated that small cell population in A172 and GBM2 showed resistance to BiCNU chemotherapy. The BiCNU-resistant cancer cells derived from GBM contained subpopulation with highly expressed CD133 cell surface marker, and this subpopulation can capable of regenerating the tumors *in vivo*. These findings may provide a novel evidence for the CSCs in GBM have chemotherapeutic drug resistance. Also, it will be possible to improve the therapeutic outcome of GBM and lead to better anticancer strategies.

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초록 : 다형성 교모세포종의 항생제 내성 종양 줄기세포

강미경 · 강수경*
(부산대학교 의과대학)

다형성 교모세포종은 뇌종양 가운데 가장 빈번하게 발병하는 악성종양이다. 다형성 교모세포종에 종양 줄기세포가 존재한다는 보고가 있음에도 불구하고, 항암제 내성과 종양 줄기세포 사이의 상호 연관성에 관한 연구는 아직 미비한 실정이다. 본 연구에서 다형성 교모세포종 세포주 A172 및 뇌종양 환자로부터 확립한 GBM2에 1,3-bis(2-chloroethyl)-1-nitrosourea (BiCNU)를 처리시 극소량의 세포군만이 생존하며, 이들 생존 세포군은 BiCNU 재처리에 내성을 나타내는 것으로 조사되었다. 또한 이 다형성 교모세포종 유래 BiCNU-내성세포군의 Erk 및 Akt 인산화 활성이 증가되었으며, CD133 줄기세포 표지인자를 발현하는 세포가 다량 존재하였다. 이와 아울러, 다형성 교모세포종 유래 BiCNU-내성세포를 severe combined immuno-deficient (SCID) mouse brain에 이식하였을 때 암이 형성되는 것을 관찰할 수 있었다. 이와 같은 결과는 다형성 교모세포종 유래 BiCNU-내성세포가 종양줄기세포의 능력을 가지는 것으로 생각된다. 따라서 이상의 결과는 다형성 교모세포종에 존재하는 종양줄기세포가 항암제 내성에 관여 한다는 중요한 단서를 제공해줄 수 있을 것으로 사료된다.