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Overcoming Chemo-resistance of Pediatric Ependymoma by Inhibition of STAT3 Signaling

STAT3 신호전달계 억제를 이용한 소아 상의세포종양의 항암제저항성 극복

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Overcoming Chemo-resistance of Pediatric Ependymoma by Inhibition of STAT3 Signaling

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ABSTRACT

Introduction: The long-term clinical outcome of pediatric intracranial epepdymoma is poor with high rate of recurrence. One of the main reasons for this poor outcome is the tumor's inherent resistance to chemotherapy. Signal tranducer and activator of transcription 3 (STAT3) is overactive in many human cancers, and inhibition of STAT3 signaling is an emerging area of interest in oncology. In this study, the possibility of STAT3 inhibition as a treatment was investigated in pediatric intracranial ependymoma tissues and cell lines.

Methods: STAT3 activation status was checked in ependymoma tissues. The responses to conventional chemotherapeutic agents and a STAT3 inhibitor, WP1066 in primarily cultured ependymoma cells were measured by cell viability assay. Apoptosis assays were conducted to reveal the cytotoxic mechanism of applied agents. The change of STAT3 signaling after WP1066 treatment was evaluated.

Results: High levels of phospho-STAT3 (p-STAT3) expression were observed in ependymoma tissue, especially in the anaplastic histology group. There was no cytotoxic effect of cisplatin, ifosfamide, and etoposide. Both brain tumor-initiating cells (BTICs) and bulk tumor cells (BCs) showed considerably decreased viability after WP1066 treatment. However, BTICs had fewer responses than BCs. No additive or synergistic effect was observed for combination therapy of WP1066 and cisplatin. Robust apoptosis was observed after WP1066 treatment. BTICs of other brain tumors also activated STAT3

and WP1066 effectively abrogated p-STAT3 expression. Increase of the interleukin-6 receptor (IL-6R) was observed after WP1066 treatment.

Conclusions: In this study, we observed a cytotoxic effect of STAT3 inhibitor on ependymoa BTICs and BCs. There is urgent need to develop new therapeutic agents for pediatric ependymoma. STAT3 inhibitors may be a new group of drugs for clinical application.

Keywords: ependymoma, chemo-resistance, STAT3 inhibitor
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LIST OF ABBREVIATIONS

World Health Organization (WHO)

Radiation therapy (RT)

Signal transducer and activator of transcription 3 (STAT3)

Brain tumor-initiating cell (BTIC)

Bulk tumor cell (BC)

Dimethyl sulfoxide (DMSO)

Dulbecco’s phosphate buffered saline (DPBS)

Epidermal growth factor (EGF)

Basic fibroblast growth factor (bFGF)

Dulbecco's Modified Eagle's Medium (DMEM)

Fetal bovine serum (FBS)

Neural stem cell (NSC)

Atypical teratoid rhabdoid tumor (ATRT)

4′-6-diamidino-2-phenylindole (DAPI)

Cell counting kit-8 (CCK)

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Propidium iodide (PI)

Tris-buffered saline (TBS)

Phospho-STAT3 (p-STAT3)

Interleukin 6 (IL-6)

INTRODUCTION

Ependymoma is an intra-axial brain tumor that predominantly develops in young children. In the histological grading system of brain tumors proposed by the World Health Organization (WHO), ependymomas correspond to either grade 2 (classic) or grade 3 (anaplastic variant) (1). However, the prognosis of ependymoma is dire despite its modest histological grade. Long-term survival rates of pediatric ependymoma patients range from 50 to 70% (2–5). In a recently published analysis of our patients' outcome, we observed a high rate of tumor recurrence (73% of patients). The five-year overall survival rate was only 71% (6). The poor treatment outcome of ependymoma patients urges clinicians to speculate on the legitimacy of current treatment protocols.

Current treatment protocols for intracranial ependymoma consist of a maximal surgical resection followed by local radiation therapy (RT). Surgical morbidities for radical resection of intracranial ependymomas can be considerable because many ependymomas involve brainstem and cranial nerves. Ependymoma is intrinsically resistant to chemotherapy and currently no reliable and uniform chemotherapy regimen exists for this tumor. Although some reported efficacy of high-dose chemotherapy with stem cell rescue for a small number of ependymoma patients, the limited efficacy and high toxicity of treatment need to be further verified (7). RT has a moderate effect on tumor control of intracranial ependymomas and most treatment protocols advocate early postoperative RT. However, the average age of ependymoma patients is younger than that of medulloblastoma. In our previous study, 42% of patients were under the age of three at diagnosis—the conventional age threshold to

allow safe delivery of high-dose radiation to developing brains (6). Recently, clinicians in the St. Jude Children's Research Hospital reported that early application of conformal RT, even to patients under one year, could raise the local control rate of intracranial ependymoma to over 80% (8). This should be considered as a true improvement, but RT for such a young-age group needs to be more sophisticated in application. In this context, development of a new therapeutic agent can provide a robust means to prevent surgical complications, to postpone RT until brain maturation, and to enhance the survival of the children.

Signal transducer and activator of transcription 3 (STAT3) is a crucial intracellular signal transducer that, once activated by various growth factors and cytokines, binds to DNA and guides specific gene expression (9). STAT3 protein expression has been known to be elevated in many kinds of human cancers (10). Furthermore, in some cancers such as colorectal, ovarian, and renal cell carcinomas, high STAT3 expression was associated with poor prognosis of patients (11-13). STAT3 protein has pleiotropic functions that lead to cancer cell survival, cell cycle progression, angiogenesis, and immune modulation through turning on and off the transcription of many genes (9). Especially, STAT3 may play a role in augmenting chemo-resistance in many types of human cancers (14–16). Interruption of STAT3 signaling by STAT3 siRNA *in vitro* reversed chemo-resistance to cisplatin in human ovarian cancer cell lines (15).

Many kinds of STAT3 inhibitors have been developed (9, 10). Each of the inhibitors blocks a certain stage of the complex STAT3 signaling pathway and

some of the agents have shown efficacy in suppressing tumor growth and enhancing survival of *in vivo* tumor models. Recently, human clinical trials using oral STAT3 inhibitors were launched and are ongoing for advanced and refractory cancers (17). Therefore, the STAT3 inhibitor may be a potentially promising therapeutic agent for intrinsically chemo-resistant tumors like intracranial ependymoma.

During the last decade, tumor-initiating cells have been found in many solid cancers including brain tumors (18). These tumor-initiating cells have demonstrated stem cell–like characteristics in gene expression, sphere-formation in specialized culture media, and serial tumor formation after transplantation into immune-deficient mice. Tumor-initiating cells may have an important role in chemo-resistance, tumor dormancy, and metastasis (19, 20). Interestingly, in colon cancer cells, $ALDH^+/CD133^+$ tumor-initiating cells showed higher STAT3 activation levels and these cells were sensitive to STAT3 inhibition (21). Brain tumor–initiating cells (BTICs) are also found in ependymoma (22). In this study, the potential of a STAT3 inhibitor, WP1066, to suppress ependymoma cell growth and to overcome chemo-resistance was evaluated in the context of BTICs and bulk tumor cells (BCs) to test the therapeutic potentials of STAT3 inhibitors for pediatric ependymoma.

MATERIALS AND METHODS

1. Human brain tissues

Fresh human brain tumor specimens were collected from patients undergoing respective surgery at the Seoul National University Children's Hospital. Eligible patients or their parents provided written informed consent to provide the tumor tissues. The Institutional Review Board of the Seoul National University Hospital approved the tissue banking and this study protocol. None of the patients received neo-adjuvant therapies. Non-tumor brain tissues were obtained from patients receiving surgery for pharmacologically intractable epilepsy. Tissues used for experiments are as follows: epilepsy (N = 5), ependymoma (WHO grade 2; N = 6), and anaplastic ependymoma (WHO grade 3; N = 6). Tissue selection was determined by tissue availability and performed by a person blinded to the treatment outcome of the patients.

2. Reagents

WP1066, a STAT3 inhibitor was purchased from Skelleckchem (Huston, TX). Chemotherapeutic agents cisplatin, ifosfamide, and etoposide were obtained from Sigma-Aldrich (St. Louis, MO). All the agents were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Stock solutions were made at a concentration of 50 mM in DMSO and stored at −20ºC.

3. Primary cell culture

Fresh tumor tissues were mechanically chopped in Dulbecco's phosphate buffered saline (DPBS; Invitrogen, Grand Island, NY) without calcium and magnesium before enzymatic digestion. Within 6 h after resection, tissues were washed and enzymatically dissociated into single cells and filtered through a 40 μm filter. Red blood cells were removed. Live cells were seeded at a standard density of 2×10^5 cells/cm^2 Primary tumor cell cultures were maintained in Neurobasal Medium (Invitrogen) containing 2 mM L-glutamine, N2 supplement, B27 supplement, and 20 ng/ml of human recombinant epidermal growth factor (EGF: Chemicon, Temecula, CA) and a basic fibroblast growth factor (bFGF; Chemicon). To generate tumor-spheres, the cells were seeded at a standard density of 2×10^5 cells/cm^2 at each passage. Spheres were maintained and subcultures were performed accordingly. All primarily cultured cells were used for *in vitro* experiments in less than four cell passages. For BC culture, tumor cells were transferred to Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and penicillin-streptomycin (×1 final concentration; Invitrogen). All cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere. Neural stem cells (NSCs; F3 cell line) were cultured in the same media condition.

Because we used only early-passage (<4) cells for all *in vitro* experiments, four different primary ependymoma cell lines were used (designated as SNU-EP1, SNU-EP2, SNU-EP3, and SNU-EP4, respectively). SNU-EP4 cells were from WHO grade 2 ependymoma (patient age 25 months). SNU-EP1, SNU-EP2, and SNU-EP3 cells originated from anaplastic ependyoma (patient age

16 years, three years, and five years, respectively). The diagnosis of other brain tumors includes glioblastoma (N = 2), atypical teratoid rhabdoid tumor (ATRT; N = 2), choroid plexus papilloma (N = 1), and pilocytic astrocytoma (N = 1).

4. In vitro sphere formation assay

For tumor-sphere formation assay, dissociated cells (1×10^3 cells, N=3) were plated onto poly-L lysine-coated 8-well plates (Nunc, Rochester, NY) in serum-free Neurobasal medium with B27 supplement (Invitrogen) for two to seven days. The number of tumor-spheres with a diameter > 40 μm were counted with an inverted microscope (Leica Microsystem, Wetzlar, Germany). All experiments were performed in triplicate.

5. Characterization of spheroid cells

For characterization of tumor-spheres, spheroid cells were fixed in 2% paraformaldehyde. Immunofluorescence staining was performed using the following antibodies: Nestin (Chemicon; 1:200) or Musashi (Neuromics, Bloomington, MN; 1:100) by immunofluorescent staining. The secondary antibodies Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit (Invitrogen; 1:200) were used. The cells were mounted with an anti-fading solution containing 4′-6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using a confocal microscope (Zeiss, Oberkochen, Germany). All experiments were conducted in triplicate.

6. Cell viability assay

The cytotoxic effect of WP1066, cisplatin, ifosfamide, or etoposide was determined using the colorimetric cell counting kit-8 (CCK; Dojindo Molecular Technologies, Gaithersburg, MD) that shows good correlation with the [^{3}H]-thymidine incorporation assay. Dissociated spheres were plated in 96-well plates in 100 μL of serum free media supplemented with growth factors or DMEM media supplemented with FBS, at a density of 5×10^3 cells/well. F3 cells used as a control cell for detecting cytotoxic effect to normal stem cells. After exposure to drugs (at concentrations from 0 μM to 50 μM) for 48 hrs, the CCK reagent was added to each well. The absorbance of the samples against a background control was measured using a Microplate Reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 450 nm. The viability of non-treated cells was regarded as 100%. IC50 values represent the drug concentrations required to inhibit 50% of cell viability and were calculated with a linear regression line of the plot of percentage inhibition (23).

7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Cells were grown on glass coverslips. After treatment with WP1066 for 48 h, TUNEL staining was performed using Click-iT TUNEL Alexa Fluor 594, as recommended by the supplier (Invitrogen). Cells were counterstained with Hoechst dye for the nuclei and fluorescent images were obtained using a

confocal microscope (Zeiss). The results are from three independent experiments. The apoptotic cell rate was determined according to the formula: (number of apoptotic cell with red staining / total number of cells with DAPI staining) × 100%.

8. Annexin V assay

In order to evaluate the stages of apoptosis, cells were stained with the Annexin V-FITC Apoptosis Detection kit (BD Bioscience, San Jose, CA) and counterstained with propidium iodide (PI; BD Bioscience) after a 24h WP1066 treatment. Fluorescent intensities were determined by a FACSCalibur flow-cytometer (Becton Dickinson, San Jose, CA). The experiment was repeated three times.

9. Western blotting

Total proteins were extracted from brain tissues and cells using protein lysis buffer (Cell Signaling Technologies, Danvers, MA). Equal amounts of protein (30 μg) were mixed with NuPAGE sample buffer (Invitrogen), boiled for 10 min at 70°C. Then the samples were separated by sodium NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose i-blot gel transfer stack (Invitrogen) using i-blot system (Invitrogen). After the membrane had been blocked with Tris-buffered saline (TBS) containing 5% non-fat dry milk, the membranes were treated with anti-STAT3, anti-phospho-STAT3 (p-STAT3; Cell signaling Technologies; 1:1000), anti-Cleaved Caspase-3 (Cell signaling Technologies; 1:1,000), anti-Bcl-xl (Cell Signaling Technologies; 1:1,000),

anti-Bcl2 (Abcam, Cambridge, MA; 1:1,000), anti-interleukin 6 (IL-6; Abcam; 1:1,000), anti-IL6R (Thermo Fisher Scientific, Rockford, IL; 1:1,000), anti-ABCC4 (Cell Signaling Technologies; 1:1,000), and anti-β-Actin (Sigma-Aldrich; 1:5,000). Chemiluminescence reagent (Invitrogen) and were exposed to the film. The blots were developed with enhanced blot densities of the proteins were normalized to the levels of internal β-actin expression and were represented as the relative intensity values.

10. Statistical analysis

Unpaired t-test was applied to compare continuous variables between two groups., A Kruskal-Wallis test was used for comparison of continuous variables in three groups. IBM SPSS version 19.0 software (IBM, Armonk, NY) was used for statistical analyses.

RESULTS

STAT3 activation in ependymoma tissues

The status of STAT3 activation was assessed using western blotting in fresh-frozen tissues of ependymoma (WHO grade 2) and anaplastic ependymoma (WHO grade 3). Brain tissues from focal cortical dysplasia patients were used as controls. The STAT3 expression was highly elevated in tumor tissues compared with controls (Fig 1A). Tumor tissues also showed high level of STAT3 activation with thick bands for p-STAT3. Three of six cases of ependymoma showed high p-STAT3 expression and five of six cases of anaplastic ependymoma revealed high p-STAT3 expression. Semi-quantitative values of band intensity of STAT3 were 0.308 ± 0.142, 0.738 ± 0.833, and 0.730 ± 0.505 for brain tissue, ependymoma, and anaplastic ependymoma, respectively. The values for p-STAT3 were 0.144 ± 0.297, 0.470 ± 0.345, and 0.520 ± 0.312 in the same order. There was a marginal difference in STAT3 band intensities among the three categories ($p = 0.0546$; Kruskal-Wallis test), but a significant difference was noted in p-STAT3 band intensity ($p = 0.0298$; Kruskall-Wallis test; Fig. 1B).

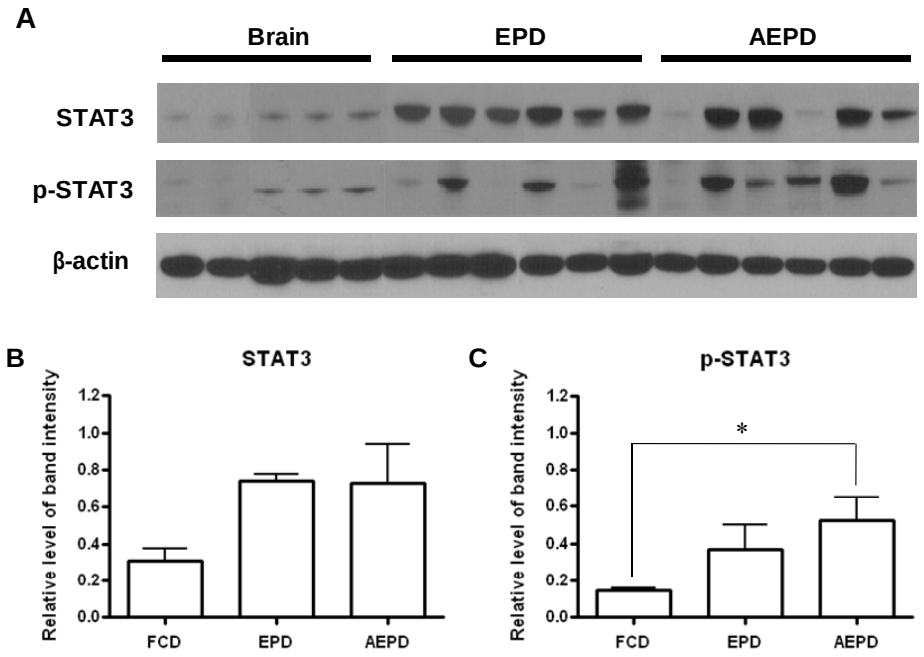

Fig. 1 (A) STAT3 expression in ependymoma tissues. Abundant expression of STAT3 and p-STAT3 was observed in WHO grade 2 ependymoma (EPD) and WHO grade 3 anaplastic ependymoma (AEPD) compared to brain tissues (Brain). (B and C) In semi-quantitative measurement, the band intensity of STAT3 was marginally different in the three categories (P = 0.0546), but there was a significant difference of p-STAT3 expression among the groups (P = 0.0298).

Culture of BTICs and BCs

Four fresh tumor tissues were chopped, dissociated, and cultured into two different conditions. One is a culture method optimized for BTICs and the other is a serum-added condition for BCs. BTICs formed multiple characteristic spheroids, whereas BCs showed an elongated morphology (Fig. 2). The same morphological characteristics were also observed for other brain tumor cells (glioblastomas, ATRTs, choroid plexus papilloma, and pilocytic astrocytoma). In immunofluorescence, BTICs were strongly positive for neural stem cell markers, Nestin and Musashi (Fig. 3). These markers were not expressed in BCs (data not shown).

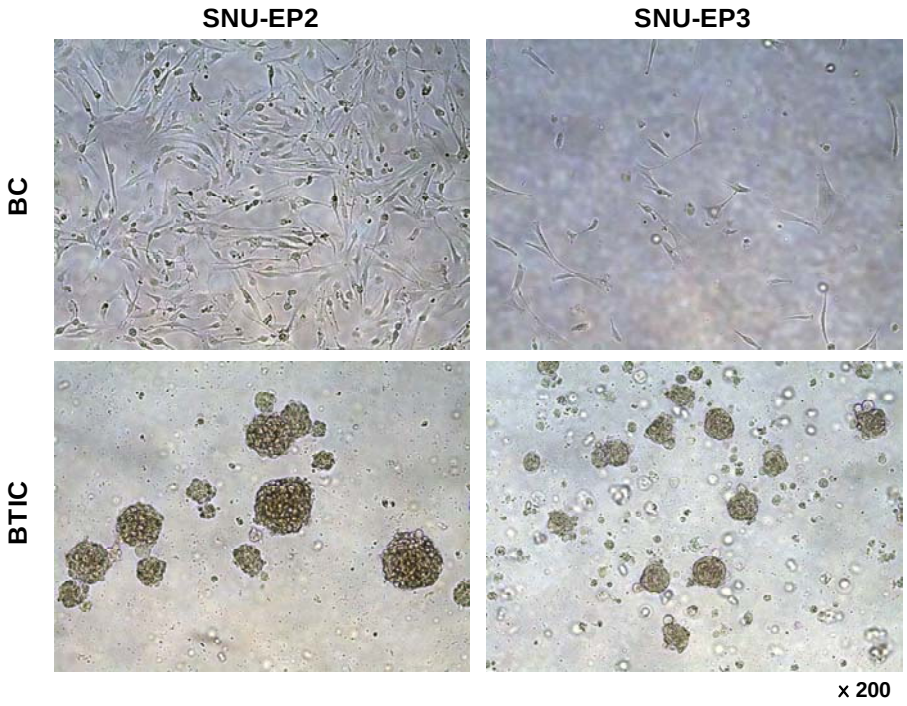

Fig. 2. Distinct morphological divergence of ependymoma cells in two different culture conditions. Upper row is bulk tumor cells (BCs) cultured in serum-containing media and the lower row is brain tumor-initiating cells (BTICs) cultured in serum-free media (×200).

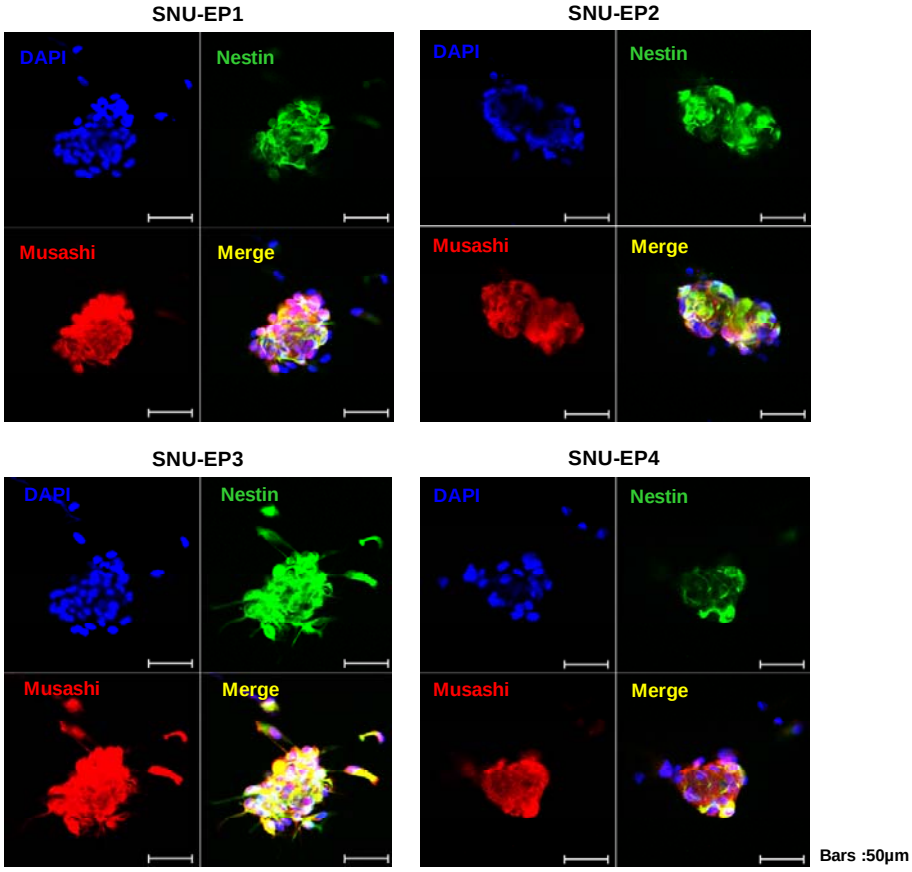

Fig. 3. Expression of stem cell markers in ependymoma BTICs. Immunofluorescence staining showed intense expression of nestin and musashi in the tumor spheres of ependymoma BTICs.

Sensitivity of ependymoma cells to various chemotherapeutic agents

To assess the cytotoxic effect of chemotherapeutic agents currently used for intracranial ependymomas, cell viability assay was conducted for SNU-EP1 cells after treatment of cisplatin, ifosfamide, etoposide, or WP1066 in serial concentrations. Drugs were delivered to either BTICs or BCs of SNU-EP1 cells, respectively. There was virtually no cytotoxic effect of cisplatin and ifosfamide. The IC_{50} values of cisplatin for BCs and BTICs of SNU-EP1 cells were 95.5 ± 19.9 μM and 213.6 ± 149.6 μM, respectively. The IC_{50} values of ifosfamide for BCs and BTIC were 152.1 ± 47.3 μM and 113.0 ± 12.3 μM, respectively (Fig. 4A and 4B). Etoposide showed a modest decrease of cell viability. IC_{50} values were 49.7 ± 2.9 μM for BCs and 49.4 ± 1.5 μM for BTICs (Fig. 4C). In contrast, WP1066 showed smaller IC_{50} value 39.4 ± 2.2 μM for BCs than all chemotherapeutic agents tested above. The IC_{50} value of WP1066 for BTICs was similar to that of etoposide, 55.9 ± 1.9 μM (Fig. 4D). The IC_{50} values of drugs for SNU-EP1 cells were summarized in Table 1.

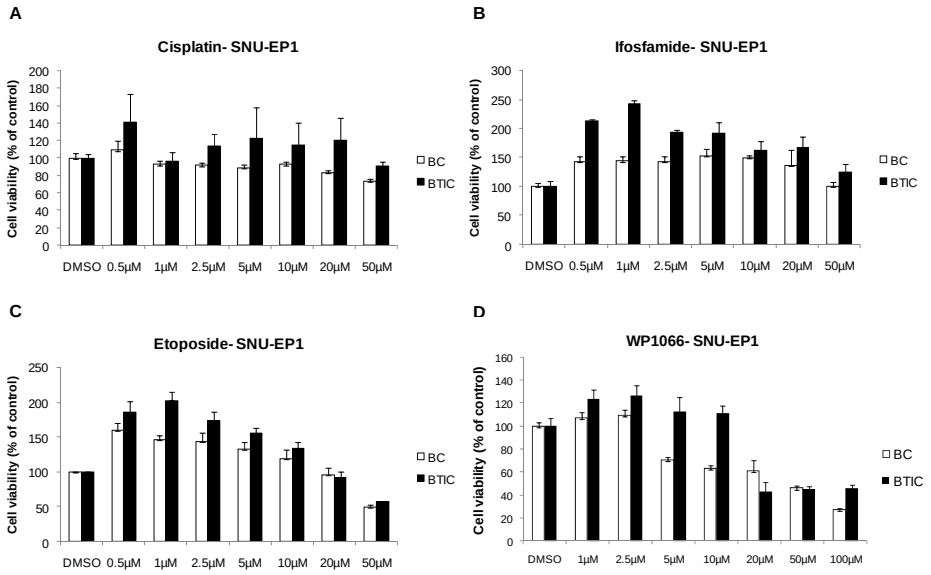

Fig. 4. Responses of ependymoma cells (SNU-EP1) to conventional chemotherapeutic agents and WP1066. WP1066 showed better cytotoxic effects to both BTIC and BC of SNU-EP1 cells than cisplatin and ifosfamide.

Table 1. Comparison of the half-maximal inhibitory concentration (IC_{50}) values for drugs in ependymoma cells (SNU-EP1)

Drugs	BC	BTIC
Cisplatin	95.5 ± 19.9	213.6 ± 149.6
Ifosfamide	152.1 ± 47.3	113.0 ± 12.3
Etoposide	49.7 ± 2.9	49.4 ± 1.5
WP1066	39.4 ± 2.2	55.9 ± 1.9

Bulk tumor cell (BC), brain tumor-initiating cell (BTIC), The IC_{50} value, relative to untreated control. IC_{50} ± SD (μM).

WP1066 treatment to neural stem cells and ependymoma cells

The IC_{50} value of WP1066 to a neural stem cell line, F3 cells, was 32.1 ± 0.9 μM (Fig. 5A). For three other primary ependymoma cell lines (SNU-EP2, SNU-EP3, and SNU-EP4 cells), treatment of WP1066 resulted in cytotoxicity to both BTICs and BCs (Fig 5B, 5C, 5D). The IC_{50} values for these cell lines are summarized in Table 2. Inhibition of BTICs was more effective in SNU-EP3 cells. In contrast, cytotoxicity to BCs was more represented in SNU-EP2 and SNU-EP4 cells. Interestingly, BTICs of SNU-EP3 cells escaped the suppressive effect of WP1066 at higher concentrations.

Table 2. Comparison of the half-maximal inhibitory concentration (IC_{50}) for WP1066 in various primary ependymoma cell lines

Cells	BC	BTIC
SNU-EP2	3.1 ± 0.5	12.3 ± 1.3
SNU-EP3	20.4 ± 1.9	5.6 ± 0.8
SNU-EP4	1.3 ± 0.1	8.9 ± 5.2

Bulk tumor cell (BC), brain tumor-initiating cell (BTIC), The IC_{50} value, relative to untreated control. IC_{50} ± SD (μM).

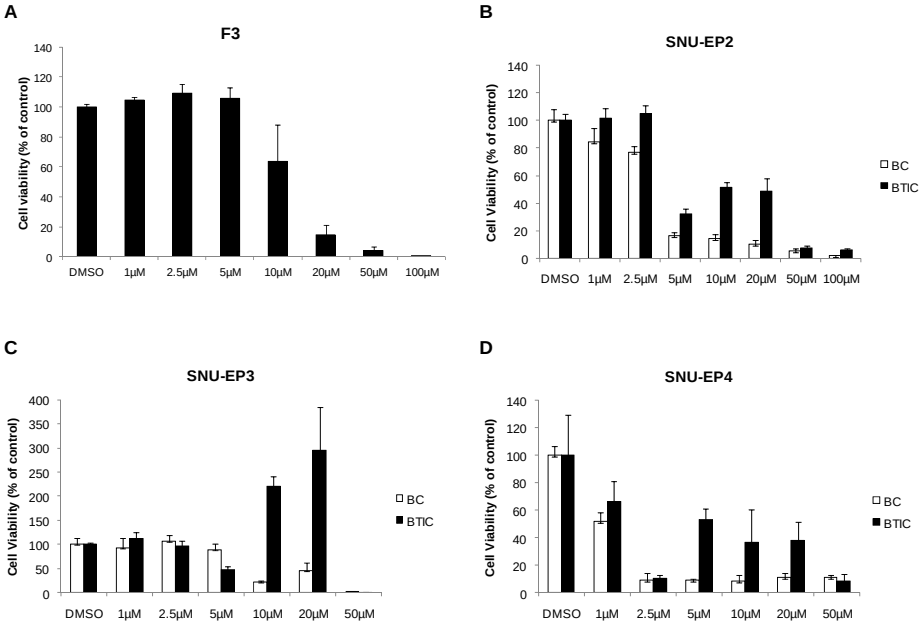

Fig. 5. Responses of F3, a neural stem cell line and ependymoma primary cell lines to WP1066. (A) WP1066 effectively reduced cell viability of F3. (B–D) WP1066 showed stable cytotoxic effects on BCs of SNU-EP2, SNU-EP3, and SNU-EP4 cell lines. BTICs of these cell lines were also suppressed by WP1066, but their responses were variable in the cell lines tested.

Combination treatment of WP1066 and conventional chemotherapeutic agents

Cell viability was assessed after various combinations of WP1066 and conventional chemotherapeutic agents in two ependymoma cell lines (SNU-EP1 and SNU-EP2). As shown in Table 1, cisplatin and ifosfamide alone showed no cytotoxic effects and etoposide exhibited modest cytotoxic effects on SNU-EP1 cells. Combination of conventional agents with WP1066 yielded no additive or synergistic effect for all cell lines compared with the response to WP1066 alone. Indeed, cytotoxic effects of WP1066 seemed to be hampered by adding conventional agents. There were no meaningful differences in the response patterns between day 2 and day 3 after drug treatment.

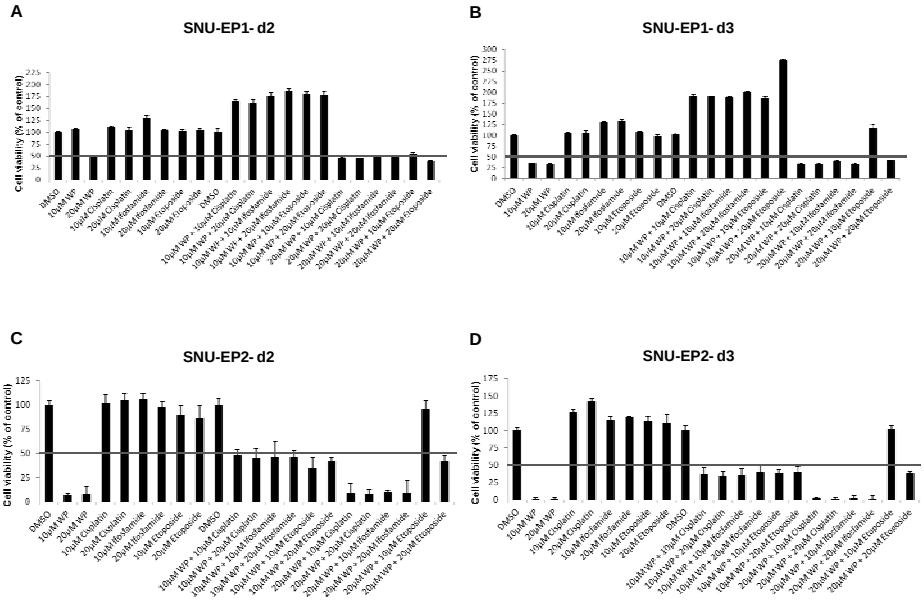

Fig. 6. Combination treatment of WP1066 and conventional chemotherapeutic agents. Cell viability was assessed in day 2 (d2) and day 3 (d3) for each cell line. There was virtually no additive or synergistic effect of drug combination compared with the response to WP1066 alone. Horizontal bars (grey color) represent 50% cell viability.

Increased apoptosis after WP1066 treatment

TUNEL and Annexin V assays were performed for SNU-EP1 and SNU-EP2 cells to verify whether WP1066 induces apoptosis in ependymoma cells. In TUNEL assay, apoptotic cells increased dramatically after WP1066 treatment (Fig. 7). The proportion of apoptotic cells were 0.40 ± 0.53 % for control cells and 85.5 ± 11.8 % for WP1066-treated SNU-EP1 cells ($p = 0.0063$; unpaired t-test). In SNU-EP2 cells, the proportions were 1.31 ± 1.13 % in control cells and 90.6 ± 4.30 % in WP1066-treated cells ($p = 0.0004$; unpaired t-test).

In Annexin V assay, augmentation of early apoptosis was more prominent than increase of late apoptosis after WP1066 treatment (Fig. 8). In SNU-EP1 cells, the proportions of early apoptotic cells were 0.96 ± 0.27 % in control cells and 7.7 ± 2.66 % in WP1066-treated cells ($p = 0.0468$; unpaired t-test). There was no significant difference in the proportions of late apoptotic cells (9.87 ± 0.80 % in control cells and 13.1 ± 1.65 % in WP1066-treated cells; $p = 0.0590$; unpaired t-test). In SNU-EP2 cells, the proportions of early apoptotic cells were 2.55 ± 0.45 % in control cells and 18.8 ± 1.56 % in WP1066-treated cells ($p = 0.0016$; unpaired t-test). The proportions of late apoptotic cells were 4.30 ± 0.32 % in control cells and 12.6 ± 0.24 % in WP1066-treated cells ($p < 0.0001$; unpaired t-test).

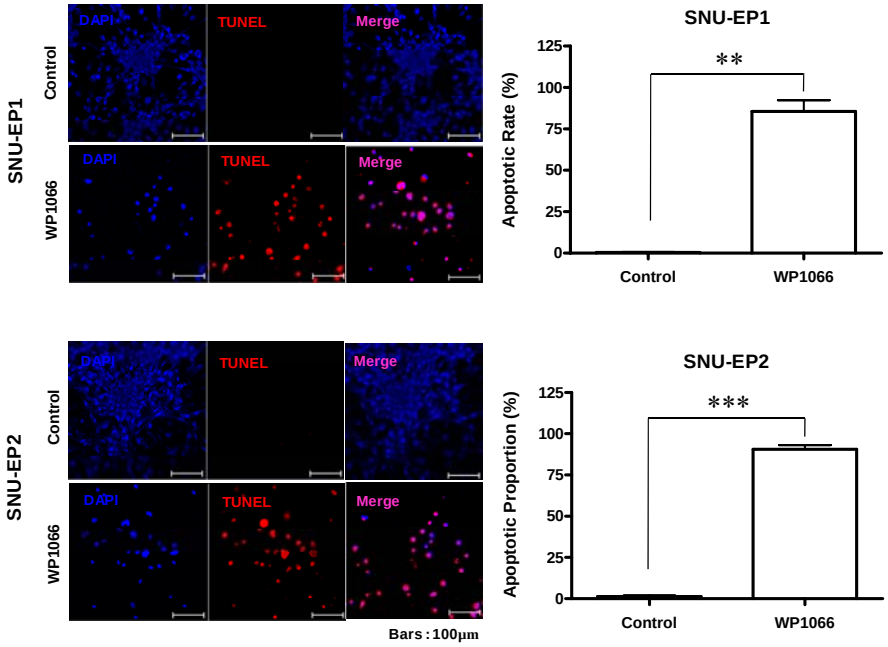

Fig. 7. TUNEL assays in two ependymoma cell lines after WP1066 treatment. A dramatic increase of apoptotic cell proportion was observed in SNU-EP1 and SNU-EP2 cells.

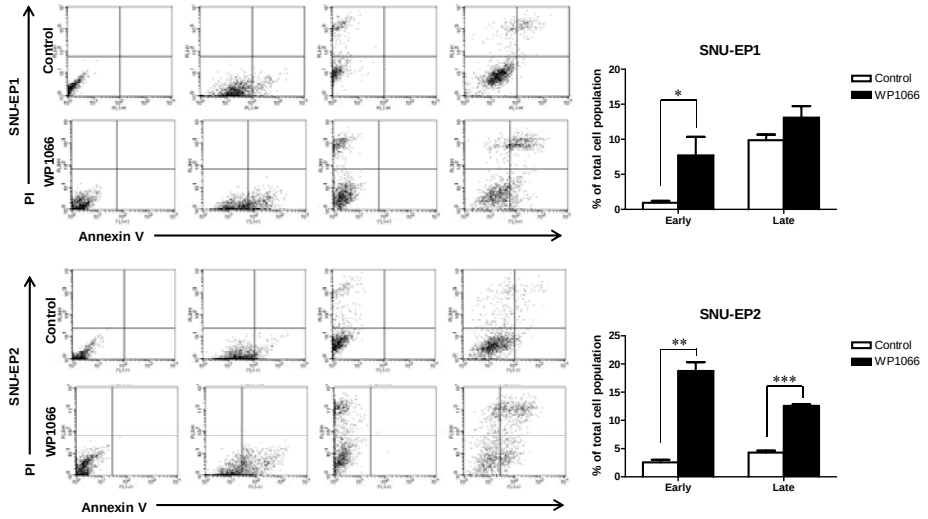

Fig. 8. Annexin V assays in two ependymoma cell lines after WP1066 treatment. Increase of apoptotic cell proportion was more prominent in early apoptosis.

Expression of apoptosis-related proteins after WP1066 treatment

We observed the expression of cleaved caspase-3 and anti-apoptotic factors, Bcl-xl and Bcl-2, after WP1066 treatment in SNU-EP1 and SNU-EP2 cells. At 72 h after WP1066 treatment, p-STAT3 expression was nadir in both cell lines. Cleaved caspase-3 expression was paradoxically decreased, indicating that a caspase-independent mechanism may be working in the apoptosis of ependymoma cells after STAT3 inhibition. The level of Bcl-xl was not changed. Instead, there was a decrease of Bcl-2 expression in both cell lines. However, the decrease of Bcl-2 expression was observed 72 h after WP1066 treatment (Fig. 9).

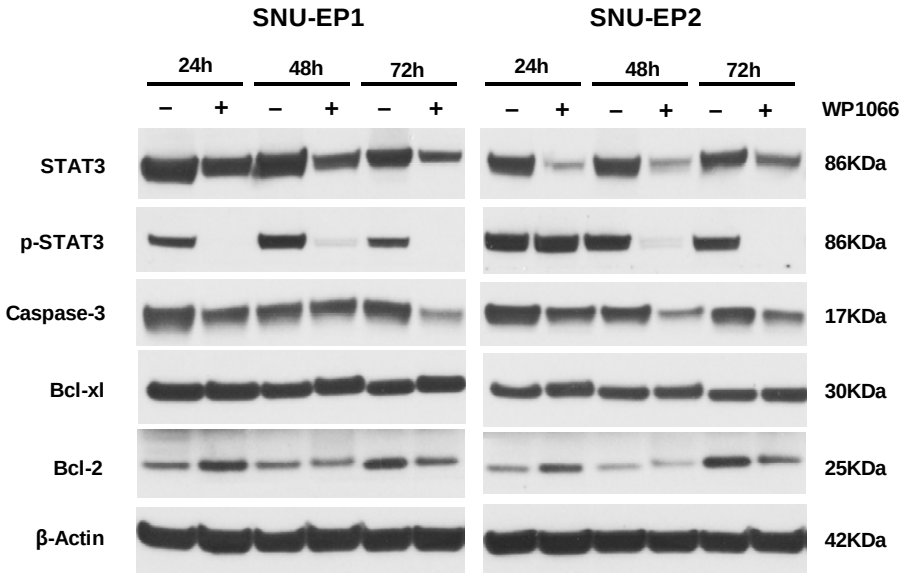

Fig. 9. Expression of apoptosis-related proteins. 72 h after the WP1066 treatment, an anti-apoptotic factor Bcl-2 decreased in SNU-EP1 and SNU-EP2 cells.

Changes in the STAT3 signaling pathway after WP1066 treatment

We applied WP1066 to other kinds of brain tumors and explored the expression of p-STAT3, IL-6, IL-6 receptor (IL-6R), and ATP-binding cassette sub-family C member 4 (ABCC4). IL-6R constitutes a major upstream pathway of STAT3 signaling in which IL-6 acts as a ligand. ABCC4 is one of the proteins involved in multi-drug resistance. WP1066 effectively suppressed the expression of p-STAT3 in BTICs of glioblastomas, ATRTs, pilocytic astrocytoma as well as of ependymoma (Fig. 10). IL-6R expression was increased after WP1066 treatment in the BTICs of ependymomas, glioblastomas, ATRTs, and pilocytic astrocytoma. Increase of IL-6R expression was not evident in BCs of ependymoma and choroid plexus papilloma. A temporary decrease of IL-6 expression was observed within 48 h after WP1066 treatment in ependymoma BTICs, but its expression increased after 54 h. This can reflect autocrine activation of IL-6 after some interval in BTICs. The response of ABCC4 to WP1066 treatment was not consistent in various cell lines.

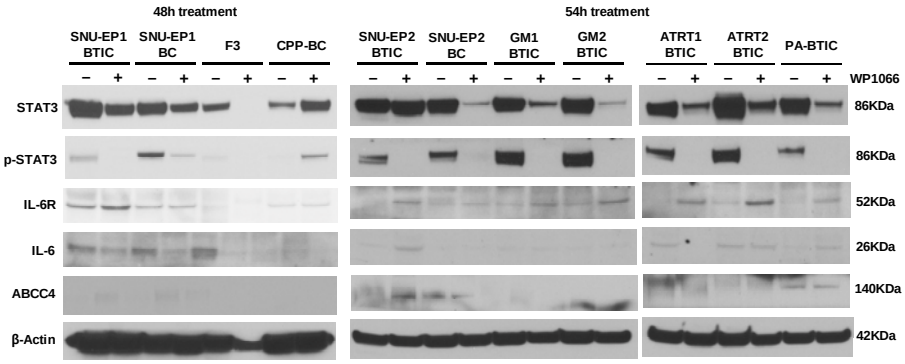

Fig. 10. Changes of STAT3 signaling pathway after treatment of WP1066. p-STAT3 expression was suppressed in BTICs of glioblastoma, ATRT, pilocytic astrocytoma as well as ependymoma. There was an increase of IL-6R after WP1066 treatment in BTICs of various tumors. CPP= choroid plexus papilloma, GM= glioblastoma, PA= pilocytic astrocytoma

DISCUSSION

STAT3 is an important cellular signal transducer interconnecting many receptors, signaling pathways, and target genes. *In vitro* studies showed that activation of STAT3 pathway leads to enhanced cell survival, cell cycle progression, and cellular migration, all of which are cardinal features of malignancy. Indeed, many human cancers showed over-expression of STAT3 and activation of its signaling that has prognostic correlation in many instances (11, 13). Therefore, suppression of the activated STAT3 pathway has been a topic of investigation for many types of cancer. Inhibition of STAT3 is possible by administration of specific siRNA, decoy oligonucleotide, or synthetic or natural inhibitors (10, 24). There are many kinds of STAT3 inhibitors and WP1066 has been known as a potent inhibitor of STAT3 pathway (25). STAT3 is constitutively activated in malignant glioma and WP1066 have shown growth-suppressing ability in U87-MR and U373-MG cells *in vitro* and *in vivo* (26). The cellular effects of STAT3 pathway inhibition are diverse, but the induction of apoptosis is regarded as the main consequence (25) Therefore, STAT3 inhibitors can acts as a cytotoxic agent. Silencing of STAT3 resulted in reduction of glioma cell infiltration into the mouse brain, thereby prolonging the survival of the animal model (27). This finding presents another promising role of STAT3 inhibitors as an anti-invasive agent for malignant brain tumors.

In this study, a STAT3 inhibitor, WP1066 treatment led to robust suppression of ependymoma cell growth. Conventional chemotherapeutic

agents (cisplatin, ifosfamide, and etoposide) showed insufficient cytotoxic effects to ependymoma cells *in vitro*. It has been postulated that STAT3 signaling is deeply involved in the acquisition of chemo-resistance of tumor cells (28, 29). In gastric cancer cells, it was shown that treatment of STAT3 siRNA increased sensitivity to cisplatin in drug-resistant cell populations (16). Monoclonal antibody to IL-6, siltuximab, increased the cytotoxicity of paclitaxel in drug-resistant ovarian carcinoma cell lines by inhibiting the STAT3 signaling (30).

However, we observed no synergistic effect of combined treatment of WP1066 and chemotherapeutic agents. Limited efficacy to a certain group of patients and the emergence of resistance in initially responsive patients are major obstacles frequently encountered during application of new molecular-targeted agents. At present, we may have to proceed on trying a single STAT3 inhibitor or to search for an optimal combination of drugs for ependymoma to maximize the effect of STAT3 inhibitors.

We found that the responses to WP1066 differed in several ependymoma cell lines. Especially, some ependymoma cells (SNU-EP3 cells) escaped the suppressive effect of WP1066 even at higher concentration. Considering that we used primarily cultured tumor cells, it is possible that a high level of genetic heterogeneity confounded the experiment. Otherwise, this situation can reflect actual clinical situation more closely in which each patient (tumor) exhibits different responses to medication albeit under the same diagnosis.

The last decade witnessed the emerging concept of cancer stem cells in various solid tumors (19). The cancer stem cell concept has been criticized

mainly for the lack of out-and-out definitions and criteria, and for brain tumors, BTIC may be more appropriate term. In this study, BTICs could be separated from glioblastoma, ATRT, ependymoma, and even from low-grade tumors, such as pilocytic astrocytoma. BTICs are thought to be dormant in nature and more resistant to chemotherapy than BCs (20). Out results showed that BTICs of ependymoma were more resistant to WP1066 in three out of four ependymoma cell lines tested. Nonetheless, WP1066 showed sufficient cytotoxicity to suppress ependymoma BTICs. WP1066 also suppressed the viability of F3 cell line, a human NSC line, proving its ability to target stem cell-like cells. This fact raises some concerns about clinical application of STAT3 inhibitors, regarding the protection of normal NCS populations in young children with brain tumors. However, F3 cell line has been generated with v-myc oncogene transfection and the proliferation rate *in vitro* is much faster than expected for normal NSCs *in vivo* (31). Therefore, an *in vivo* experiment is required to confirm the effect of STAT3 inhibitors on ependymoma cells as well as on the residing normal NCSs.

Previous studies suggested that STAT3 inhibitors including WP1066 augment apoptosis of tumor cells especially through suppression of anti-apoptotic factors, such as survivin or Bcl-xl (30). We also observed a dramatic increase of apoptosis after WP1066 treatment in ependymoma cell lines. We also observed a decrease of Bcl-2 rather than of Bcl-xl. However, the decrease of Bcl-2 expression was evident at 72 h after WP1066 treatment, whereas robust apoptosis was observed in TUNEL staining and Annexin V assay within 24–48 hrs. Therefore, the apoptosis mechanism induced by STAT3

inhibition may be diverse and depend on cellular context.

In our study, STAT3 activation was observed in other malignant brain tumors, such as glioblastoma and ATRT. Although limited to only one case, increased p-STAT3 expression was not found for choroid plexus papilloma cells. Emerging evidence suggests that glioma stem-like cells are dependent on STAT3 signaling for tumor progression (32). Therefore, STAT3 may be a common target for a variety of high-grade brain tumors.

At present, several human clinical trials are ongoing that target refractory solid tumors with novel STAT3 inhibitors (OPB-31121 and OPB-51602) and chronic lymphocytic leukemia with an anti-protozoal medication (pyrimethamine) that has STAT3-inhibiting actions (17). In our study, we observed cytotoxic effect of STAT3 inhibitor to ependymoa BTICs and BCs. We have urgent needs to develop new therapeutic agents for pediatric ependymoma patients. STAT3 inhibitors may emerge as a new group of drugs for clinical application for pediatric intracranial ependymomas.

REFERENCES

1. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, et al. The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol. 2007 Aug;114(2):97-109.
2. McGuire CS, Sainani KL, Fisher PG. Both location and age predict survival in ependymoma: a SEER study. Pediatr Blood Cancer. 2009 Jan;52(1):65-9.
3. Shu HK, Sall WF, Maity A, Tochner ZA, Janss AJ, Belasco JB, et al. Childhood intracranial ependymoma: twenty-year experience from a single institution. Cancer. 2007 Jul 15;110(2):432-41.
4. Tamburrini G, D'Ercole M, Pettorini BL, Caldarelli M, Massimi L, Di Rocco C. Survival following treatment for intracranial ependymoma: a review. Childs Nerv Syst. 2009 Oct;25(10):1303-12.
5. van Veelen-Vincent ML, Pierre-Kahn A, Kalifa C, Sainte-Rose C, Zerah M, Thorne J, et al. Ependymoma in childhood: prognostic factors, extent of surgery, and adjuvant therapy. J Neurosurg. 2002 Oct;97(4):827-35.
6. Phi JH, Wang KC, Park SH, Kim IH, Kim IO, Park KD, et al. Pediatric infratentorial ependymoma: prognostic significance of anaplastic histology. J Neurooncol. 2012 Feb;106(3):619-26.
7. Sung KW, Lim do H, Lee SH, Yoo KH, Koo HH, Kim JH, et al. Tandem high-dose chemotherapy and autologous stem cell transplantation for anaplastic ependymoma in children younger than 3

years of age. J Neurooncol. 2012 Apr;107(2):335-42.

8. Merchant TE, Li C, Xiong X, Kun LE, Boop FA, Sanford RA. Conformal radiotherapy after surgery for paediatric ependymoma: a prospective study. Lancet Oncol. 2009 Mar;10(3):258-66.

9. Yue P, Turkson J. Targeting STAT3 in cancer: how successful are we? Expert Opin Investig Drugs. 2009 Jan;18(1):45-56.

10. Johnston PA, Grandis JR. STAT3 signaling: anticancer strategies and challenges. Mol Interv. 2011 Feb;11(1):18-26.

11. Horiguchi A, Oya M, Shimada T, Uchida A, Marumo K, Murai M. Activation of signal transducer and activator of transcription 3 in renal cell carcinoma: a study of incidence and its association with pathological features and clinical outcome. J Urol. 2002 Aug;168(2):762-5.

12. Morikawa T, Baba Y, Yamauchi M, Kuchiba A, Nosho K, Shima K, et al. STAT3 expression, molecular features, inflammation patterns, and prognosis in a database of 724 colorectal cancers. Clin Cancer Res. 2011 Mar 15;17(6):1452-62.

13. Rosen DG, Mercado-Uribe I, Yang G, Bast RC, Jr., Amin HM, Lai R, et al. The role of constitutively active signal transducer and activator of transcription 3 in ovarian tumorigenesis and prognosis. Cancer. 2006 Dec 1;107(11):2730-40.

14. Nair RR, Tolentino JH, Hazlehurst LA. Role of STAT3 in Transformation and Drug Resistance in CML. Front Oncol. 2012;2:30.

15. Han Z, Feng J, Hong Z, Chen L, Li W, Liao S, et al. Silencing of the

STAT3 signaling pathway reverses the inherent and induced chemoresistance of human ovarian cancer cells. Biochem Biophys Res Commun. 2013 May 31;435(2):188-94.

16. Huang S, Chen M, Shen Y, Shen W, Guo H, Gao Q, et al. Inhibition of activated Stat3 reverses drug resistance to chemotherapeutic agents in gastric cancer cells. Cancer Lett. 2012 Feb 28;315(2):198-205.
17. http://clinicaltrials.gov/ct2/results?term=stat3+inhibitor&Search=Search; accessed at 07/Jul/2013
18. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Identification of human brain tumour initiating cells. Nature. 2004 Nov 18;432(7015):396-401.
19. Tirino V, Desiderio V, Paino F, De Rosa A, Papaccio F, La Noce M, et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. FASEB J. 2013 Jan;27(1):13-24.
20. Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med. 2013;2(1):3.
21. Lin L, Liu A, Peng Z, Lin HJ, Li PK, Li C, et al. STAT3 is necessary for proliferation and survival in colon cancer-initiating cells. Cancer Res. 2011 Dec 1;71(23):7226-37.
22. Taylor MD, Poppleton H, Fuller C, Su X, Liu Y, Jensen P, et al. Radial glia cells are candidate stem cells of ependymoma. Cancer Cell. 2005 Oct;8(4):323-35.
23. http://www.sciencegateway.org/protocols/cellbio/drug/hcic50.htm; accessed at 22/Feb/2014

24. Sen M, Thomas SM, Kim S, Yeh JI, Ferris RL, Johnson JT, et al. First-in-human trial of a STAT3 decoy oligonucleotide in head and neck tumors: implications for cancer therapy. Cancer Discov. 2012 Aug;2(8):694-705.

25. Ferrajoli A, Faderl S, Van Q, Koch P, Harris D, Liu Z, et al. WP1066 disrupts Janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. Cancer Res. 2007 Dec 1;67(23):11291-9.

26. Iwamaru A, Szymanski S, Iwado E, Aoki H, Yokoyama T, Fokt I, et al. A novel inhibitor of the STAT3 pathway induces apoptosis in malignant glioma cells both in vitro and in vivo. Oncogene. 2007 Apr 12;26(17):2435-44.

27. Priester M, Copanaki E, Vafaizadeh V, Hensel S, Bernreuther C, Glatzel M, et al. STAT3 silencing inhibits glioma single cell infiltration and tumor growth. Neuro Oncol. 2013 Jul;15(7):840-52.

28. Alas S, Bonavida B. Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis. Clin Cancer Res. 2003 Jan;9(1):316-26.

29. Ara T, Nakata R, Sheard MA, Shimada H, Buettner R, Groshen SG, et al. Critical Role of STAT3 in IL-6-Mediated Drug Resistance in Human Neuroblastoma. Cancer Res. 2013 Jul 1;73(13):3852-64.

30. Guo Y, Nemeth J, O'Brien C, Susa M, Liu X, Zhang Z, et al. Effects of siltuximab on the IL-6-induced signaling pathway in ovarian cancer.

Clin Cancer Res. 2010 Dec 1;16(23):5759-69.

31. Kim SU, Nagai A, Nakagawa E, Choi HB, Bang JH, Lee HJ, et al. Production and characterization of immortal human neural stem cell line with multipotent differentiation property. Methods Mol Biol. 2008;438:103-21.

32. Stechishin OD Luchman HA, Ruan Y, Blough MD, Nguyen SA, Kelly JJ, et al. On-target JAK2/STAT3 inhibition slows disease progression in orthotopic xenografts of human glioblastoma brain tumor stem cells. Neuro Oncol. 2013;15:198-207.

국문 초록

서론: 소아의 두개강내 상의세포종양의 치료성적은 좋지 않으며 재발률이 높다. 이 같은 나쁜 치료성적에는 항암제에 대한 근본적인 내성이 그 원인으로 지목되고 있다. 신호전달 단백질인 STAT3 는 많은 암에서 증가되어 발현되는데 STAT3 신호전달계를 억제하는 치료법이 여러 암에 대해서 시도되고 있다. 이 연구에서는 STAT3 억제제를 이용하여 상의세포종양을 치료하는 가능성을 소아 뇌에 발생한 상의세포종양의 조직과 세포주에서 검증하고자 한다.

방법: 상의세포종양 조직에서 STAT3 활성여부를 측정하였다. STAT3 억제제인 WP1066 과 함께 기존에 사용되고 있는 항암제들에 대한 상의세포종양 세포의 생존반응을 관찰하였다. 세포사멸반응 검사를 시행하여 종양억제의 기전을 밝히고 WP1066 처치 후에 STAT3 신호전달계의 변화를 아울러 관찰하였다.

결과: 상의세포종양, 특히 역형성 종양조직에서 강한 인산화-STAT3 의 발현이 관찰되었다. Cisplatin, ifosfamide, etoposide 는 항암 효과가 거의 없었는데 반하여 WP1066 에 대하여 뇌종양줄기세포와 일반종양세포 모두 암세포의 생존이 감소되었다. 그러나 실험한 네가지 중 세가지 세포주에서 뇌종양줄기세포의 반응 정도는 일반종양세포보다 적었음을 관찰하였다. 기존 항암제들을 WP1066 에 추가하였을 때 부가적인 억제효과는 관찰되지 않았다. WP1066

처치 후에 강력한 세포사멸반응이 관찰되었다. 다른 악성뇌종양에서도 STAT3 가 활성화 되어있었으며 WP1066 은 이를 효과적으로 억제하였다. 약제 처치 후에 IL-6R 발현의 증가를 확인하였다.

결론: 이 연구에서 STAT3 억제제가 상의세포종양의 뇌종양줄기세포와 일반세포의 증식을 모두 효과적으로 억제함을 관찰하였다. 소아 상의세포종양에 대한 새로운 치료제의 개발이 시급한 상황을 고려해볼 때, 이 연구는 STAT3 억제제가 임상적용이 가능한 새로운 약제로 등장할 가능성을 제시하였다.

주요어 : 상의세포종양, 항암제저항성, STAT3 억제제

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