Down-regulation of EGFR Prolonged Cell Growth of Glioma but Did Not Increase the Sensitivity to Temozolomide

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Abstract. Background: Malignant glioma is an invasive disease of the central nervous system. One of the factors that regulate growth of these tumors is expression of epidermal growth factor receptor (EGFR) in the cells. This study investigated the effects of down-regulation of EGFR on cell proliferation, cell cycle and cytotoxicity to antineoplastic agent. Materials and Methods: A short hairpin RNA transcription vector targeting EGFR was transfected into KNS42 cells. Growth curve, cell cycle and sensitivity to temozolomide of the cells were assessed. Results: Transfection inhibited EGFR expression by 50.5%. It prolonged cell doubling time by 25.7%. However, it did not meaningfully alter the cell cycle populations nor increase sensitivity to temozolomide. Conclusion: Suppressing expression of EGFR inhibited cell proliferation. However, unlike PTEN expression or ROCK1 down-regulation, it did not alter the cell cycle or increase sensitivity to temozolomide.

Tumor therapy has developed dramatically in recent years, but still does not adequately improve the outcome of brain tumor, particularly of malignant glioma. Patients with malignant glioma are treated by surgery, radiotherapy, chemotherapy, or immunotherapy, but the median survival rate is less than 1 year (1). Glioma has the characteristic of low metastasis to tissues other than the central nervous system and tends to recur ad locum, even if the tumor has been resected. Since the speed of local mass recurrence and growth determines the progression of the disease, more efficient local therapy is required to prolong survival of the patients. In the clinical setting, direct access for local delivery of drugs or nucleic acids has been established by the advancement of neural navigation systems and other imaging techniques, and refined administration of the agents is used for the treatment of many diseases. From this standpoint, development of more effective drugs is required.

Many genes involved in tumor proliferation have been identified recently. Accordingly, drugs which target these genes have been developed and used clinically. Various studies reported that regulation of the expression of genes, such as signal transduction and activator of transcription3 (STAT3), epidermal growth factor receptor (EGFR), p53, phosphatase and tensin homolog deleted from chromosome 10 (PTEN), rho-associated kinase (ROCK) (2-5), controls cell proliferation of the tumors. Earlier, we reported that the ROCK1 down-regulation yielded increased sensitivity to an anti-neoplastic agent of malignant glioma cells (6). Recently we also reported that PTEN increased drug sensitivity (7). Regulating such genes may lead to new strategies of brain tumor therapy.

Among these, EGFR has been studied considerably in the sphere of brain tumor. EGFR is a 170-kDa receptor tyrosine kinase and binds epidermal growth factor, initiating a cascade of downstream signaling events. EGFR triggers cell proliferation, cell cycle progress, invasion, and metastasis via activation of RAS, Phosphatidylinositol 3-kinases (PI3K), or Just another kinase (JAK). EGFR also is associated with oncogenesis through various mechanisms, including amplification of the EGFR gene or deletions/mutations which activate the receptor consecutively (8). EGFR also has been reported to have an oncogenic role in 90% of primary brain tumors and effective therapy through the EGFR gene has been the subject of intense investigation (9-11).
In this study, we attempted to investigate the effects of EGFR down-regulation using a short hairpin (sh) RNA transcription vector in a KNS42 human glioma cell line which expresses EGFR. The results were compared to the effects of ROCK1 down-regulation and forced PTEN expression which were the subjects of our previous studies.

Materials and Methods

Cell lines and drugs. Human glioma cell line, KNS42 (12) was used for the study. The cell line was cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Temozolomide was kindly provided by Merck & Co., Inc. (NJ, USA) (13).

Construction of shRNA transcription vectors to EGFR and transfection into KNS42 glioma cells. sh RNA against EGFR were designed by the siRNA target finder (Ambion, TX, USA) and corresponding DNAs were synthesized (Fasmac, Atsugi, Japan) and then ligated into the BamHI and HindIII sites of the pSilencer 5.1-H1 Retro (Ambion) mammalian transcription vector. The target for EGFR was 5'AGACCATCCAGGAGGTGGCT-3'. As the control, pSilencer 5.1-H1 Retro with a scrambled sequence was also used. The vector transcribed shRNA and the product functioned as a short interferential RNA after cleavage. The vectors were transfected into cells by electroporation at 250 V with 975 μF and the cells were selected with 150 μg/ml of puromycine (CARYA, France).

Detection of EGFR protein by immunoblotting. Expression of EGFR in human glioma cell lines was confirmed by immunoblotting at 72 hours after infection. The exponentially growing cells were lysed by CHAPS buffer (Cell Signaling Technology Japan, Tokyo, Japan), and after adjustment of the amounts of protein, an equal volume of sodium lauryl sulfate sample buffer, containing Tris-pH 6.8, 2% SDS, glycerol, β-mercaptoethanol and bromophenol blue, was added. After boiling, 10 μg samples were loaded on 7.5% SDS-PAGE gels (1.5 M Tris-HCl, 10% SDS, 30% bis-acrylamide, 10% ammonium persulfate, tetramethylethylenediamine) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfont, UK). The same membranes were reacted with anti-EGFR rabbit polyclonal antibody (Cell Signaling Technology) at a 1000-times dilution. Specimens were further reacted with secondary antibodies and the signals were detected by ECL (GE Healthcare, Little Chalfont, UK). The same membranes were reacted with anti-β-actin antibody to confirm equal loading of the samples (14).

Cell growth and doubling time in vitro. Cell growth was determined by counting the cell numbers after plating. One thousand cells were plated into a 35-mm dish and the total numbers of cells after proliferation were counted. The doubling time was calculated by their growth curve (6). The doubling time by 25.7% (Figure 2).

Cell cycle analysis. Cell cycles of the transfected cells were measured by a flow cytometer (FACScan, Becton Dickinson and Company, NJ, USA) after propidium iodide (PI) staining (6). Cells (1x10^5) were dispersed with trypsin, suspended in PBS, fixed with 75% ethanol, and stained with 0.03 μM of PI with 180 units of RNaseA for 30 minutes. Populations in each cell cycle phase were analyzed by Cell Quest™ pro (ver 5.2.1, Becton Dickinson and Company).

Cell sensitivity assay to antineoplastic agent. Wild type cells and transfected cells (1x10^6) were plated in 96-well plates and exposed to different doses (7.8 μM-2 mM) of temozolomide. After 72 hours of culture, the cells were fixed with 5% glutaraldehyde and stained with 0.01% methylene blue. Dye was eluted by 0.4 N hydrochloric acid after washing the plates. The absorbance was measured 595 nm on a microplate reader (Bio-rad, CA, USA). IC_{50} (50% inhibitory concentration) values were compared (15-17).

Results

EGFR expression after shRNA transduction in KNS42 cells. First, EGFR expression in KNS42 cells was assessed. Expression was evaluated by immunoblotting and the presence of EGFR protein at 175 kDa was confirmed. Since EGFR was expressed in the cell line, mammalian shRNA transcription vector for EGFR was constructed and transduced into the cell line and amounts of EGFR expression after transduction were examined. The expression of EGFR in transfected cells with shRNA for EGFR decreased approximately 50.5% (Figure 1). Since transduction successfully down-regulated EGFR expression, the effect on cell proliferation was then investigated. EGFR down-regulation suppressed cellular growth and prolonged the doubling time to 48 hours (control 44.4 hours, wild-type 38.2 hours). Although transduction of control cells with shRNA in a scrambled sequence slightly prolonged the doubling time, EGFR down-regulation effectively reduced cell proliferation and increased the doubling time by 25.7% (Figure 2).

Cell cycle analysis. To investigate the effect on EGFR down-regulation in detail, populations of cells at each cell cycle phase were analyzed. Results demonstrated that the G1 phase population was 59.7% in the wild-type, 59.3% in the control, and 59.9% in EGFR down-regulated cells, whereas the G2/M phase population was 17.2% in the wild-type, 17.4% in the control, and 10.2% in EGFR down-regulated cells. The population pattern changed even with transduction of the control shRNA vector. When compared to the control, down-regulation of EGFR slightly reduced the G2 phase cell cycle, but the effect was equivocal (Figure 3).

Cytotoxic assay. Cell growth inhibition achieved by down-regulation of ROCK1 or forced expression of PTEN significantly conferred sensitivity of temozolomide on glioma cells in previous studies (1). To compare such an effect with that of EGFR down-regulation, sensitivity to temozolomide was measured. Cells were exposed to different concentrations of temozolomide and IC_{50} values were determined. The IC_{50} of temozolomide and IC_{50} values were determined.
values of wild-type and control cells were 583.4 μM and 731.4 μM, respectively. On the other hand, IC₅₀ value was 946.7 μM in EGFR down-regulated cells. EGFR down-regulation reduced the effect of temozolomide rather than conferring sensitivity (Figure 4).

Discussion

EGFR was targeted and the molecule was successfully down-regulated by the shRNA transcription vector. The reason for choosing KNS42 was that the effect of down-regulation could be directly compared to that of forced expression of PTEN, which was demonstrated previously with the same glioma cell line. In addition, KNS42 cells expressed EGF in our preliminary study.

Many agents targeting molecules that regulate tumor cell proliferation have been devised for tumor therapy. Among these targets, EGFR is a good choice (18-20). Approaches include EGFR signal inhibition by decoy (21), antibody that disturbs EGFR phosphorylation, decrease of EGFR through RNA interference, and suppression of signals downstream of EGFR. Antibody drugs have been used in clinical therapy. Lapatinib binds to the ATP-binding pocket of the EGFR/HER2 protein kinase domain, blocks self-phosphorylation, inhibits signals downstream and reduces cell proliferation. The agent demonstrated 24% sensitivity to breast carcinoma (22). It decreased cell proliferation by 24% in glioma in vitro (23), whereas it did not have an adequate effect in a clinical study (24) probably due to the existence of the blood brain barrier. Local therapy by direct administration of an agent into tumor tissue may have the advantage of avoiding the blood brain barrier. Several methods of transduction into the central nervous system are available for local treatment (15, 17, 25). In this study, transduction of shRNA suppressed EGFR expression. There are splicing variants in EGFR mRNA. The sequence used in the study targeted all 1-4 splicing variants of EGFR. While down-regulated cells did not demonstrate phase-specific growth arrest, down-regulation of EGFR inhibited cell proliferation and prolonged the doubling time. Glioma seldom metastasizes distantly and, in most cases, the cause of death is local expansion of the tumor mass. A decrease in proliferation of the tumor mass might result in the prolongation of survival.

Temozolomide is an alkylating agent and is utilized for adjuvant chemotherapy of brain tumors (26). In previous studies, we demonstrated that both down-regulation of ROCK1 or expression of PTEN inhibited cell growth through prolongation of the G₂ phase of the cell cycle and we observed an increased sensitivity to alkylating agents (6-7). EGFR regulates cellular function through three major pathways, PI3K/AKT, RAS/ mitogen-activated protein kinase (MAPK) and JAK/STAT. PTEN inhibits the PI3K/AKT pathway by dephosphorylating phosphatidylinositol-(3,4,5)-tri-phosphate. ROCK1 is one of the isoforms of Rho kinase in the downstream region of Rho that is activated by a signal from the G-protein-coupled receptor. Although the signal pathway of PTEN and ROCK1 were different, modulation of expression of both induced G₂ arrest in glioma cells. Unlike these molecules, such an arrest was not observed in EGFR down-regulated cells and EGFR down-regulated cells showed a slight decrease in the G₂ phase population. While the effect of the alteration is obscure, these differences in the G₂ phase may be attributable to the difference in drug sensitivity. Nonetheless, several studies have demonstrated that antibodies to EGFR or ErbB families increased sensitivity to anti-neoplastic agents (27-29). Although an alkylating agent is the first choice of drug...
for malignant glioma patients, it is important to determine whether or not there is a synergetic effect with other anti-neoplastic agents, such as gemcitabine, on the down-regulation of \(\text{EGFR}\). In addition, \(\text{EGFR}\) mutation variant III (30-33) is known to be associated with glioma progression. It has been reported that down-regulation suppressed cell growth by 15-20% and invasion by 55%, and increased the \(\text{G}_2\) phase population \((3, 8, 34)\). Further comparative study is required.

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References


![Figure 3. Effect of down-regulation of \(\text{EGFR}\) on cell cycle. Cell cycle populations of wild-type (left), control (middle), and \(\text{EGFR}\) down-regulated cells (right) were compared. \(\text{EGFR}\) down-regulation led to a slight decrease in the population of \(\text{G}_2\text{M}\) phase cells (10.2%) when compared to wild-type (17.2%) and control cells (17.4%). The horizontal axis indicates the amount of DNA, and the vertical axis indicates the cell number.](image1)

![Figure 4. Sensitivity of ROCK down-regulated cells to temozolomide. Wild-type, control and \(\text{EGFR}\) down-regulated cells were exposed to different concentrations of temozolomide for 72 hours and their sensitivities were determined by assay. The IC\(_{50}\) values of wild type, control, and \(\text{EGFR}\) down-regulated cells were 583.4 \(\mu\text{M}\), 731.4 \(\mu\text{M}\), and 946.7 \(\mu\text{M}\), respectively. KNS42 wild type (○), control (□), \(\text{EGFR}\) down-regulated (△) cells. (n=4, bars=SD).](image2)


