**Regular Article**

**Temozolomide Induces Endocytosis of EGFRvIII via p38-Mediated Non-canonical Phosphorylation in Glioblastoma Cells**

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The ligand-induced internalization of epidermal growth factor receptor (EGFR) is generally considered to attenuate downstream signaling via its endosomal degradation. However, the endocytosis of an oncogenic EGFR variant III (EGFRvIII) is impaired, which leads to persistent signaling from the cell surface, thereby promoting the proliferation and survival of glioblastoma multiforme (GBM) cells. Cellular stress triggers the non-canonical endocytosis-recycling of EGFR by p38-mediated phosphorylation. In the present study, we used temozolomide (TMZ), the standard chemotherapeutic agent for the treatment of GBM patients, to examine whether EGFRvIII is controlled by a non-canonical mechanism. TMZ triggered the endocytic trafficking of serine phosphorylated EGFRvIII. Moreover, phosphorylation and endocytosis were abrogated by the selective p38 inhibitor SB203580, but not gefitinib, indicating that EGFRvIII is recruited to p38-mediated non-canonical endocytosis. The combination of TMZ and SB203580 also showed potential inhibitory effects on the proliferation and motility of glioblastoma cells.

**Key words** epidermal growth factor receptor (EGFR); EGFR variant III (EGFRvIII); p38; temozolomide; endocytosis; glioblastoma

**INTRODUCTION**

Glioblastoma multiforme (GBM) is the most aggressive brain cancer in adults and has a poor prognosis.1–3 Uncontrolled proliferation, apoptosis resistance, robust angiogenesis, and genomic instability are the characteristics that make this cancer difficult to treat.1 The standard treatment for GBM is surgery followed by radiotherapy and chemotherapy using temozolomide (TMZ), a DNA alkylating agent that triggers temozolomide (TMZ) and SB203580 were purchased from Merck KGaA (Darmstadt, Germany). A phos-tag ligand was obtained from Aapie Ltd. (Darmstadt, Germany). The antibodies against total EGFR (1005) and actin (C-11) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-EGFR (Ser-1015) was generated using a rabbit-immunospot array assay on a chip (ISAAC) system.12 The latter route is similar to that observed in cellular stress responses, in which serine (Ser)/threonine (Thr) residues in the C-terminal region of EGFR are highly phosphorylated by p38.13–15 EGFR variant III (EGFRvIII), the most common mutant in GBM cells, is incapable of binding ligands because of the loss of the extracellular region, but harbors constitutive kinase activity.5,7,16,17 EGFRvIII is also internalized; however, instead of being transported to the lysosome, it is mainly returned to the plasma membrane.6,18 The direct binding of the ubiquitin ligase c-Cbl with phosphorylated Tyrosine (Tyr)-1045 is crucial for the lysosomal degradation of wild-type ligand-bound EGFR.19 In contrast, EGFRvIII mainly binds indirectly with c-Cbl via Grb2, resulting in impaired endocytosis and lysosomal degradation.18,20 Therefore, EGFRvIII increases tumorigenicity due to prolonged activation on the cell surface.7,8,21 However, it currently remains unclear whether EGFRvIII is regulated by a non-canonical mechanism via stress-induced p38 activation.

Therefore, we herein investigated the non-canonical endocytosis of EGFRvIII in U87MG human glioblastoma cells. Under TMZ-induced stress conditions, EGFRvIII underwent endocytosis through p38-mediated phosphorylation. Moreover, the combination of TMZ and p38 inhibition decreased cell proliferation and migration.

**MATERIALS AND METHODS**

**Antibodies and Reagents** Phospho-specific antibodies against EGFR (Tyr-1068) and p38 (Thr-180/Tyr-182) and anti-early endosome antigen 1 (EEA1) antibody (C45B10) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Anti-EGFR (Ser-1015) was generated using a rabbit-immunospot array assay on a chip (ISAAC) system.12 Antibodies against total EGFR (1005) and actin (C-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Gefitinib, an EGFR-tyrosine kinase inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). TMZ and SB203580 were purchased from Merck KGaA (Darmstadt, Germany). A phos-tag ligand was obtained from

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Wako Pure Chemical Corporation (Osaka, Japan). All chemical inhibitors were dissolved in dimethyl sulfoxide (DMSO).

**Cell Cultures** Human U87MG glioblastoma cells overexpressing wild-type EGFR (U87MG-wt) and EGFRvIII (U87MG-vIII) were kindly gifted by Profs. Webster K. Cave-nee (University of California San Diego) and Dr. Motoo Nagane (Kyorin University). Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2mM t-glutamine, 100 U/mL peni-cillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂.

**Western Blotting** Whole-cell lysates were prepared using lysis buffer (25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.7), 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, and 20 mM β-glycerophosphate). Lysates were then mixed with the same volume of sample buffer (100 mM Tris–HCl (pH 6.8), 2.0% sodium dodecyl sulfate (SDS), 10% glycerol, 70 mM DTT, and 0.10% bromophenol blue) and heated at 95°C. Cell lysates were resolved by 6.5–10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P transfer membrane. Bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) (DAKO, Glostrup, Denmark) diluted in phosphate buffered saline (PBS) and rinsed with PBS. Cells were then incubated with primary antibodies for 45 min and washed with PBS. We used the EGFR antibody, which recognizes the C-terminal domain, to detect EGFRvIII. Coverslips were incubated with an Alexa Fluor 555-conjugated goat anti-rabbit IgG antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Thermo Fisher Scientific) for 45 min followed by washing with PBS. Primary and secondary antibodies were diluted in PBS with 0.5% BSA. Coverslips were then inverted onto a glass slide with Slowfade Gold Antifade Reagent with the 4'-6-diamidino-2-phenylindole (DAPI) nuclear stain (Thermo Fisher Scientific). Fluorescence was analyzed using LSM700 confocal microscopy (Zeiss, Oberkochen, Germany).

**Cell Viability Assay** Cells were seeded onto a 96-well plate and then incubated at 37°C in 5% CO₂. After overnight culture, cells were pretreated for 30 min with 10 µM SB203580 followed by 100 µM TMZ for three days. Cells were then subjected to a viability assay using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Rockville, MD, U.S.A.).

**Migration Assay** Cells were seeded onto a 3.5-cm dish. Following treatment for 72 h, cell migration was observed using a time-lapse live cell imaging system (Carl Zeiss Cell Observer). Images were captured every 5 min for 3 h. The accumulated migrating distance of 30 cells was calculated by Imaged software.

**RESULTS**

**Anisomycin-Induced Non-canonical Phosphorylation of EGFR in U87MG Cells** We investigated whether non-canonical internalization occurred in U87MG cells overexpressing wild-type EGFR (U87MG-wt) and EGFRvIII (U87MG-vIII) upon stimulation with anisomycin, a protein synthesis inhibitor that strongly activates p38. Anisomycin rapidly induced the phosphorylation of EGFR and EGFRvIII at Ser-1015 (p5-EGFR), a residue that induces p38-dependent clathrin-mediated endocytosis (Fig. 1A). SB203580, a specific inhibitor of p38, completely blocked p5-EGFR expression in U87MG-wt and U87MG-vIII cells, whereas gefitinib, an EGFR tyrosine kinase inhibitor, did not. Similarly, immunoblotting using the Phos-tag gel revealed a marked mobility shift in the majority of EGFRvIII proteins that was dependent on p38 upon the stimulation with anisomycin (Fig. 1B).

To investigate the internalization of EGFR and EGFRvIII, cells were stained with p5-EGFR (red) and total EGFR (green) antibodies in the immunofluorescence analysis. EGFR and EGFRvIII both re-localized from the plasma membrane to the perinuclear region upon the stimulation with anisomycin (Fig. 1C). Furthermore, p5-EGFR was markedly increased by anisomycin, and completely merged with total EGFR, indicating that internalized EGFRs are phosphorylated at Ser-1015. No signal was detected in parental U87MG cells, indicating the specific staining of EGFR and p5-EGFR antibodies (data not shown). Collectively, these results demonstrated that EGFRvIII and EGFR are targeted for non-canonical internalization in glioblastoma cells.

**Induction of Non-canonical Regulation of EGFR by TMZ** We then examined the effects of TMZ on the TK-independent non-canonical regulation of EGFR. The TMZ treatment for 48 h increased p38 activation and p5-EGFR in U87MG-wt cells (Fig. 2A) and U87MG-vIII cells (Fig. 2B).
in a concentration-dependent manner. Similar results were obtained as shifted bands in the Phos-tag immunoblot analysis with the total EGFR antibody (Fig. 2C). Moreover, the pS-EGFR antibody selectively recognized only the shifted bands upon the treatment with TMZ (Fig. 2C). Since TMZ at 100 µM was sufficient to induce pS-EGFR, we used this concentration in subsequent experiments. These results demonstrated that TMZ induced the non-canonical phosphorylation of both EGFR and EGFRvIII.

**Endocytosis of EGFRvIII via TMZ-Induced p38 Activation**

A time–course analysis clearly showed p38 phosphorylation with the total EGFR antibody (Fig. 2C). Moreover, the pS-EGFR antibody selectively recognized only the shifted bands upon the treatment with TMZ (Fig. 2C). Since TMZ at 100 µM was sufficient to induce pS-EGFR, we used this concentration in subsequent experiments. These results demonstrated that TMZ induced the non-canonical phosphorylation of both EGFR and EGFRvIII.

![Graph showing endocytosis of EGFRvIII via TMZ-Induced p38 Activation](image)

**Effects of p38 Inhibition on Cell Migration**

The present results demonstrated that p38 activation is crucial for the TMZ-induced non-canonical activation of EGFRvIII. Therefore, we attempted to elucidate the effects of p38 inhibition in combination with TMZ on the proliferation and motility of U87MG-vIII cells. As shown in Fig. 4A, TMZ together with SB203580 reduced cell proliferation significantly more than TMZ or SB203580 alone. Using a time-lapse live cell imaging
system, we found significantly increased migration distances for 3 h in the cells pre-treated with TMZ for 3 d (Fig. 4B). Moreover, although SB203580 did not affect basal migration, it significantly suppressed TMZ-induced migration (Fig. 4B). These results suggest that combination therapy has the potential to overcome TMZ resistance in glioblastoma cells overexpressing EGFRvIII.

DISCUSSION

The activation of EGFR signaling occurs at the plasma membrane after ligand binding. The removal of EGFR from the cell surface by internalization for lysosomal degradation is considered to be the mechanism underlying the down-regulation of growth factor signaling. Cellular stress has been shown to promote non-canonical EGFR internalization and recycling in ligand- and TK-independent manners. Furthermore, EGFRvIII was spontaneously internalized, but at a slower rate than that of unliganded wild-type EGFR. Nevertheless, strong p38 kinase activity induced the phosphorylation of Ser-1015, which rapidly removed EGFRvIII from the cell surface (Fig. 1C). In non-small cell lung cancer (NSCLC) cells subjected to cisplatin-induced DNA damage, the phosphorylation of Ser-1015 also triggered the non-canonical internalization of EGFR harboring primary and secondary activating mutations. In the present study, we demonstrated that TMZ induced the phosphorylation of Ser-1015 in both wild-type and truncated EGFR via p38 activation in GBM cells (Figs. 2, 3). Although its physiological functions have not yet been elucidated, these results suggest that the non-canonical internalization of EGFR plays a role in the development of resistance to agents that induce DNA damage.

GBM is highly heterogeneous and generally consists of cells with different genetic alterations. Therefore, a single targeted therapy is not sufficient to inhibit this aggressive cancer. Previous studies reported the development of resistance to TMZ therapy. This limitation of current therapeutic options becomes a challenge in the treatment of GBM; therefore, combination therapy is needed to sensitize GBM to this DNA alkylating agent. We herein propose drug combination therapy with TMZ plus a p38 inhibitor for EGFRvIII-positive GBM cells. The cytotoxicity of TMZ is mainly due to the O6-methylguanine adduct that mispairs with thymine during DNA replication, which activates apoptotic signaling. The present results demonstrated that TMZ at 100 µM, a concentration that induces the endocytosis of EGFRvIII, was sufficient to inhibit the proliferation of U87MG-vIII cells. Therefore, TMZ may inhibit proliferation through its DNA-modifying activity while simultaneously inducing resistance signaling via p38 activation. The combination of TMZ with SB203580 enhanced anti-proliferative activity.

p38 was initially considered to function as a tumor suppressor in tumorigenesis due to its role in adaptation to stress conditions; however, many studies demonstrated that it also

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Fig. 2. Temozolomide-Induced Non-canonical Phosphorylation of EGFR
(A) U87MG-wt and U87MG-vIII cells were treated with various concentrations of TMZ for 72 h. (A, B) Whole-cell lysates were analyzed by normal immunoblotting with antibodies against pS-EGFR, EGFR, pp38, and actin. (C) Whole-cell lysates were separated by Zn2+ Phos-Tag and immunoblotted with anti-EGFR and pS-EGFR antibodies.
promoted cancer by enhancing migration, survival, or resistance to anticancer drugs.\textsuperscript{35,36} The inhibition of p38 activation significantly suppressed the migration of BRAF mutant melanoma cells.\textsuperscript{37} Since p38 contributes to a wide range of cellular functions,\textsuperscript{35} therapeutic strategies that target this protein kinase may be an alternative to the inhibition of U87MG-vIII cell migration, particularly in the presence of TMZ. The present results showed that TMZ-induced p38 activation is critical for glioblastoma cell migration (Fig. 4B). Consistent with the present results on proliferation and migration (Fig. 4), a phase I/II clinical trial on a p38 inhibitor (LY2228820) in combination with TMZ and radiotherapy is now being conducted to estimate the progression-free survival rate of adult GBM patients.\textsuperscript{35}

It is interesting that EGFR knockout mice are lethal with severe developmental defects, whereas mice with EGFR kinase-dead mutant have some disorders in the eye and skin, but can survive well. These observations clearly demonstrated the kinase-independent functions of EGFR. Recent advances have also shown that cellular stresses induce the kinase-independent functions of EGFR.\textsuperscript{10} For example, endosomal EGFR has been shown to be involved in the initiation of autophagy by serum starvation and anti-cancer agents. Although the mechanism of the inhibitory effect of TMZ on cell proliferation and migration has not been clarified, it is expected that the role of p38 in regulating non-canonical EGFR activity in cancer cells will be elucidated for future molecular targeted cancer therapy.

In summary, we herein demonstrated that TMZ-induced cellular stress through p38 regulated receptor endocytosis and migration in human glioblastoma cells overexpressing EGFRvIII. These results provide insights into the molecular mechanisms underlying TMZ-induced non-canonical phosphorylation. Moreover, combination therapy is regarded as

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**Fig. 3. Temozolomide-Induced Endocytosis of EGFRvIII**

(A, B) U87MG-vIII cells were treated with 100\textmu M TMZ for indicated times. (C–E) Cells were pretreated with or without 10\textmu M SB203580 (D, E) and 1\textmu M gefitinib (F) for 30 min, followed by 100\textmu M TMZ for 48 h. (A, D) Whole cell lysates were analyzed by immunoblotting with antibodies against pS-EGFR, pY-EGFR, EGFR, pp38, and actin. (B, C, E, F) The localization of total EGFR, pS-EGFR and EEA1 were analyzed by immunofluorescence. Scale bar = 10\textmu m. (Color figure can be accessed in the online version.)
an effective approach for overcoming acquired resistance to EGFRvIII-targeted therapies. Further analysis is needed to evaluate the potential efficacy by establishing EGFRvIII xenograft models.

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Conflict of Interest The authors declare no conflict of interest.

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Fig. 4. Roles of p38 in the Proliferation and Migration of U87MG-vIII Cells
(A) U87MG-vIII cells were pretreated with 10µM SB203580 for 30min followed by 100µM TMZ for 48h. Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8). (B) U87MG-vIII cells were pretreated with 10µM SB203580 and 100µM TMZ for 72h, and cell migration was then observed using the time-lapse imaging system for 3h. Accumulated migrating distances were shown in box and whisker plots. ns, not significant; **p≤0.01, by an analysis of variance followed by the Tukey–Kramer test.
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