EGFRvIII Is Expressed in Cellular Areas of Tumor in a Subset of Glioblastoma

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Abstract

Epidermal growth factor receptor variant III (EGFRvIII) is a tumor-specific cell surface antigen often expressed in glioblastoma and has drawn much attention as a possible therapeutic target. We performed immunohistochemistry on histology sections of surgical specimens taken from 67 cases with glioblastoma, isocitrate dehydrogenase-wild type, and evaluated the morphological characteristics and distribution of the EGFRvIII-positive tumor cells. We then evaluated the localization of EGFRvIII-expression within the tumor and peritumoral areas. EGFRvIII immunopositivity was detected in 15 specimens taken from 13 patients, including two recurrent specimens taken from the same patient at relapse. Immunofluorescence staining demonstrated that EGFRvIII-positive cells were present in cells positive for glial fibrillary acidic protein (GFAP), and some showed astrocytic differentiation with multiple fine processes and others did not show. The EGFRvIII-positive cells were located in cellular areas of the tumor, but not in the invading zone. In the two recurrent cases, EGFRvIII-positive cells were markedly decreased in one case and retained in the other. With regard to overall survival, univariate analysis indicated that EGFRvIII-expression in patients with glioblastoma was not significantly associated with a favorable outcome. Double-labeling immunofluorescence staining of EGFRvIII and GFAP showed that processes of large, well differentiated, GFAP-positive glia extend to and surround less differentiated, EGFRvIII-positive glial cells in cellular areas of tumor. However, in the tumor periphery, EGFRvIII-positive tumor cells were not observed. This finding suggests that EGFRvIII is involved in tumor proliferation, but that invading glioma cells lose their EGFRvIII expression.

Key words: glioblastoma, EGFRvIII, morphology, distribution, prognosis

Introduction

Glioblastoma is the most common histopathological type of primary brain tumor in adults. Despite recent application of multidisciplinary treatment involving a combination of surgical resection, chemotherapy, and radiation therapy, the prognosis of patients with glioblastoma is still poor and the median overall survival remains 14–15 months. At present, various potential therapeutics, including Rindopepimut – an anti-epidermal growth factor receptor variant III (EGFRvIII) peptide vaccine – are being assessed in clinical trials.

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EGFRvIII, the product of the EGFR gene with an in-frame deletion of exons 2–7 (del 2–7 EGFR, or ∆EGFR), is a tumor-specific cell surface antigen with a molecular mass of 145 kDa. EGFRvIII constitutively activates the STAT and PI3K-Akt pathways and promotes angiogenesis and tumor growth. In glioblastoma, it has been shown that EGFRvIII is expressed in a subset of primary tumors, with molecular profiles of EGFR amplification and absence of isocitrate dehydrogenase 1 (IDH1) mutations.

In this study, we performed immunohistochemistry using a recently available antibody specific for EGFRvIII on histology sections of surgical specimens taken from patients with glioblastoma, IDH-wild-type, in order to evaluate the morphological characteristics and distribution of EGFRvIII-positive tumor cells, and also the significance of EGFRvIII expression.
Materials and Methods

Patients
We reviewed the medical records of 67 consecutive patients (34 males, 33 females; age at surgery, mean = 64.5 years) who were admitted to the Department of Neurosurgery, Niigata University Medical and Dental Hospital, Japan, between 2011 and 2017, and diagnosed pathologically as having glioblastoma, IDH-wild-type. In accordance with the methods stipulated in the WHO Classification of Tumors,25 immunohistochemistry for IDH1 and DNA sequencing for IDH1 and IDH2 were performed, as described previously.26 The clinical profiles of the patients are summarized in Tables 1 and 2.

In cases #11 and #13 (Table 2), tumor recurrence was detected by follow-up magnetic resonance imaging (MRI), and the additional resection of the tumor was performed 5 and 10 months after surgery, respectively.

Postoperative contrast enhancement MRI was performed within 48 h of surgery. Gross total resection, subtotal resection, and partial resection were defined as no residual tumor, 90–99% removal, and <90% removal of the tumor, respectively.

This study was approved by the Ethics Committee of Niigata University School of Medicine and written informed consent for use of the resected tissues for research purposes was obtained from all patients.

Histology and immunohistochemistry
Surgical specimens were fixed with 20% buffered formalin and embedded in paraffin. Histopathological examination was performed on serial 4-µm-thick sections stained with hematoxylin and eosin (HE) and the Klüver–Barrera (KB) method. Selected sections were processed for immunohistochemistry using the methods described elsewhere.26 As the primary antibodies, we used two mouse monoclonal antibodies against EGFRvIII (clone L8A4; Absolute Antibody, Oxford, UK; dilution 1:200, pretreated by heating and clone DH 8.3; Millipore, Temecula, CA, USA; 1:200), and mouse monoclonal antibodies against human IDH1 R132H (clone H09; Dianova, Hamburg, Germany; 1:100), and vimentin (clone V9, Dako, Glostrup, CA, USA; 1:200), and mouse monoclonal antibodies against glial fibrillary acidic protein (GFAP: Dako, Glostrup, Denmark; 1:1500). We evaluated the distribution and morphology of the vimentin-positive tumor cells. Diagnosis of glioblastoma was based on the criteria listed in the WHO Classification of Tumors of the Central Nervous System.25 MGMT immunoreactivity was evaluated in representative areas of the tumors showing the characteristic features defining their histological grades as described previously using a cutoff of 30%.20 Staining for EGFRvIII was considered positive when staining (usually punctate) was evident in the cytoplasm of tumor cells.

Multiplex ligation-dependent probe assay
To investigate the amplification of EGFR, we performed multiple ligation-dependent probe assay.27 We used five samples, “normal cortex”, “glioblastoma, IDH-wildtype showing EGFRvIII immunoreactivity (patient #10 in Table 2)”, other two “glioblastoma IDH-wildtype”, which showed no EGFRvIII immunoreactivity, and “anaplastic oligodendroglioma”. DNA was extracted from paraffin-embedded tumor tissue using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Detection of EGFR amplification was performed by a multiplex ligation-dependent probe assay

Table 1 Clinical profiles of patients in each group

|                         | EGFRvIII Positive | EGFRvIII Negative | P  |
|-------------------------|-------------------|-------------------|----|
| Total                   | 13                | 54                |    |
| Age                     | 66.5              | 63.8              | 0.223 |
| Female sex              | 4 (30.8%)         | 28 (51.9%)        | 0.540 |
| Laterality              |                   |                   |    |
| Right                   | 8 (61.5%)         | 27 (50.0%)        | 0.736 |
| Left                    | 4 (30.8%)         | 20 (37.0%)        | 0.302 |
| Bilateral               | 1 (7.7%)          | 7 (13.0%)         | 0.237 |
| Tumor location          |                   |                   |    |
| Frontal                 | 5 (38.5%)         | 26 (48.1%)        | 0.758 |
| Temporal                | 3 (23.1%)         | 14 (25.9%)        | 0.999 |
| Parietal                | 1 (7.7%)          | 3 (5.6%)          | 0.999 |
| Occipital               | 2 (15.4%)         | 1 (1.9%)          | 0.094 |
| Insular                 | 1 (7.6%)          | 2 (3.7%)          | 0.482 |
| Corpus callosum         | 1 (7.6%)          | 2 (3.7%)          | 0.482 |
| Multiple                | 0                 | 6 (11.1%)         | 0.588 |
| Surgery                 |                   |                   |    |
| GTR                     | 3 (23.1%)         | 13 (24.0%)        | 0.999 |
| STR                     | 8 (61.5%)         | 16 (29.6%)        | 0.051 |
| PR                      | 2 (15.4%)         | 25 (46.3%)        | 0.059 |
| Radiation therapy       | 13 (100%)         | 52 (96.3%)        | 0.999 |
| Temozolomide            | 12 (92.3%)        | 51 (94.4%)        | 0.999 |
| Bevacizumab             | 5 (%)             | 7 (13.0%)         | 0.204 |
| Additional surgery      | 2 (%)             | 5 (9.3%)          | 0.614 |
| MGMT positivity         | 7 (53.8%)         | 24 (44.4%)        | 0.556 |

GTR: gross total resection, PR: partial resection, STR: subtotal resection.
assay (SALSA MLPA probemix P105-D2 Glioma-2, MRC Holland, Amsterdam, The Netherlands).

**Western blotting**
To confirm the reliability of the EGFRvIII antibody, we performed western blotting of tissue from one glioblastoma patient (#10 in Table 2), one recurrent glioblastoma patient and one patient diagnosed as having an anaplastic oligodendroglioma with IDH-mutation. Proteins extracted from fresh-frozen surgical specimens were separated by 10% SDS-PAGE, using the methods described previously. As the primary antibodies, we used a rabbit polyclonal antibody against EGFR (Millipore, Burlington, MA, USA, dilution 1:1000), a mouse monoclonal antibody against EGFRvIII (clone L8A4; Absolute Antibody, 1:200), a rabbit polyclonal antibody against phosphor-EGFR pTyr1086 (Invitrogen, Carlsbad, CA, USA, 1:1000) and antibody against β-actin (anti-β-Actin-HRP-DirecT, MBL, Nagoya, Japan, 1:2000).

**Double-labeling immunofluorescence**
A double-labeling immunofluorescence study was performed to assess the expression of EGFRvIII and GFAP in the tumor cells, using the paraffin sections described above. As the primary antibodies, we used the same mouse monoclonal antibody against EGFRvIII (1:50), and rabbit polyclonal antibody against GFAP (1:1000) that we had used for immunohistochemistry at different concentrations. The secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (Jackson ImunoResearch; 1:200). 4,6-Diamidino-2-phenylindole staining agent was used to visualize the nuclei by microscopy. The images were acquired using an Olympus FV1200 Laser Scanning Confocal Microscope (Olympus Life Science, Tokyo, Japan).

**Statistical analysis**
Data analysis was perfomed using GraphPad Prism 7.0 statistical software (http://graphpad-prism.software.informer.com/7.0). Overall survival (OS) was calculated from the time of surgery until death, or last follow-up according to the Kaplan–Meier method with long-rank test for comparison of survival between patients with EGFRvIII-positive and EGFRvIII-negative glioblastomas (Table 1). Unpaired Student’s t-test was used for continuous variables, and Fisher’s exact test for categorical variables. Differences at \( p \leq 0.05 \) were considered as statistically significant.

**Results**

**EGFR amplification and EGFRvIII detection**
Epidermal growth factor receptor amplification was only detected in the specimen taken from patient #10, which showed EGFRvIII-positivity (Fig. 1A). In two cases of glioblastoma, IDH-wildtype without confirmed EGFRvIII-positivity and one case of anaplastic oligodendroglioma, IDH-mutant did not demonstrate EGFR amplification compared with normal cortex. Western blotting using the monoclonal antibody against EGFRvIII was performed, and we confirmed the presence of a single band at around 145 kDa (Fig. 1B) in protein taken from tumor in

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**Table 2 Clinical and surgical characteristics of 13 patients harboring EGFRvIII-positive glioblastomas**

| Patient | Age | Sex | OP    | MGMT | P1 (%) | P2 (%) | RT | TMZ | Rec | Treatment at recurrence | OS (m) | D/A |
|---------|-----|-----|-------|------|--------|--------|----|-----|-----|-------------------------|--------|-----|
| 1       | 59  | M   | GTR   | –    | 36     | 0      | +  | +   | PD  | Surgery, TMZ             | 62     | A   |
| 2       | 81  | M   | PR    | +    | 19     | –      | +  | +   | PD  | TMZ                     | 14     | D   |
| 3       | 75  | F   | STR   | –    | 34     | –      | +  | +   | PD  | TMZ, Bev                 | 34     | D   |
| 4       | 59  | M   | GTR   | +    | 48     | 0      | +  | +   | PD  | TMZ, RT                  | 13     | D   |
| 5       | 67  | M   | STR   | –    | 56     | 0      | +  | +   | PD  | TMZ, Bev                 | 36     | D   |
| 6       | 67  | F   | PR    | –    | 31     | –      | +  | +   | PD  | TMZ                     | 21     | D   |
| 7       | 81  | M   | STR   | +    | 42     | –      | +  | –   | PD  | TMZ, RT, Bev             | 13     | D   |
| 8       | 72  | M   | STR   | –    | 21     | –      | +  | +   | PD  | –                       | 8      | D   |
| 9       | 76  | F   | STR   | –    | 100    | 0      | +  | +   | PD  | –                       | 16     | A   |
| 10      | 48  | M   | STR   | +    | 39     | –      | +  | +   | PD  | Bev                     | 12     | A   |
| 11      | 69  | M   | STR   | +    | 94     | 0      | +  | +   | PD  | Surgery                 | 9      | D   |
| 12      | 62  | M   | STR   | +    | 49     | 0      | +  | +   | PD  | –                       | 5      | A   |
| 13      | 69  | M   | GTR   | +    | 26     | –      | +  | +   | PD  | Bev, TMZ                | 15     | A   |

A: alive, Bev: bevacizumab, D: dead, GTR: gross total resection, OS: overall survival, P1: EGFRvIII-positive ratio in the cellular component, P2: EGFRvIII-positive ratio in the tumor periphery, PD: progressive disease, PR: partial resection, SD: stable disease, STR: subtotal resection, Rec: recurrence, RT: radiation therapy, TMZ: temozolomide.
Fig. 1  (A) Multiple ligation-dependent probe assay. Horizontal axis means EGFR exon number and vertical axis means signal intensity. One “glioblastoma, IDH-wildtype” patient (case #10 in Table 2) showed higher signal intensity compared with other specimens. (B) EGFR antibody recognized 170 and 145 kDa bands, which correspond to wtEGFR and EGFRvIII, respectively in case #10 and a recurrent glioblastoma, IDH-wildtype case, but not in an anaplastic oligodendroglioma, IDH-mutant case. EGFRvIII antibody recognized the only the 145 kDa band. (C–F) Representation of the features of case #5. (C) T1-weighted magnetic resonance image with contrast enhancement (MRI-T1,CE) demonstrates a large tumor in the right occipital lobe. (D) A histology section of the resected brain. Klüver–Barrera stain. The central portion of the tumor indicated by square 1 shows a high nuclear concentration, whereas the peripheral portion indicated by square 2 exhibits relative myelin pallor. (E) A serial section immunostained with the EGFRvIII antibody. EGFRvIII immunoreactivity is seen in the cellular portion of tumor. (F) Higher-power magnification views of the cellular area (upper panels; corresponding to square 1 in D and E) and the peripheral portion (lower panels; corresponding to square 2 in D and E) of the tumor. In the cellular areas, highly cellular tumor cells show immunoreactivity for vimentin, EGFRvIII and GFAP, having multiple fine processes, whereas in the peripheral portion the infiltrating tumor cells show immunoreactivity for vimentin and GFAP, but not for EGFRvIII. Scale bars = 50 µm. (G) Representation of the features of cases #1, #9, #11, #12. The tumor cells expressing EGFRvIII existed in the central portion of the tumor (upper panels), but not in the peripheral portion (lower panels). The tumor cells heterogeneously expressed EGFRvIII in the tumor and the positive ratio at the site with the most positive cells was different between tumors (23–100%).
a patient with glioblastoma, IDH-wildtype patient (case #10), and recurrent glioblastoma, IDH-wildtype. Two bands were noted for EGFR, the lower band at 145 kDa corresponding with EGFRvIII consistent with previous reports. Only one band at 170 kDa, corresponding with wildtype EGFR, was found in tissue taken from a patient with anaplastic oligodendroglioma, IDH-mutant.

**EGFRvIII expression**

Epidermal growth factor receptor variant III immunoreactivity was detected in 15 specimens, including two specimens at the time of tumor recurrence, taken from 13 patients (Table 2: 19.4% of the 67 patients). The tumor cells heterogeneously expressed EGFRvIII in the tumor and the positive ratio at the site with the most positive cells was different between tumors (median 39%, range 19–100%, Fig. 1G and Table 2), whereas there was no staining (0%) in the periphery of six assessible EGFRvIII gliomas. EGFRvIII distribution was successfully assessed in six specimens (cases #1, 4, 5, 9, 11, 12) because the tumor resection range was wide and the storage condition was optimal. The tumor cells expressing EGFRvIII existed in cellular areas of the tumor, but not in cells of the invading zone (Figs. 1C–1G, 2A–2G). Cellular areas were defined as areas of the tumor with high cellularity, usually near the tumor core and away from the tumor periphery and perinecrotic areas (Figs. 1D–1F, 2B–2F). Two different antibodies against EGFRvIII were used, but similar staining was observed (Fig. 2C–2F). Positivity for both EGFRvIII and GFAP were seen in cellular areas with cells showing prominent features of astrocytic differentiation having multiple fine processes (Figs. 1C–1G, 2A–2G). EGFRvIII staining was not evident in the invading zone of the same specimens (Figs. 1E–1G, 2B–2G). Double-labeling immunofluorescence staining for EGFRvIII and GFAP showed that EGFRvIII and GFAP do not colocalize, but that processes of large, well differentiated, GFAP-positive glia extend to and surround less differentiated, EGFRvIII-positive glial cells in cellular areas of tumor (Fig. 2G).

Two patients whose tumor initially showed EGFRvIII immunoreactivity relapsed and underwent second surgeries. EGFRvIII-positive cells were markedly decreased at relapse in one case (#11) and retained in the other (#13) (Fig. 3A), and EGFRvIII-positivity was confirmed by western blot only in an area where viable cells remained (Fig. 3B).

**Prognosis**

There was no difference in median OS between patients with EGFRvIII expression (1.83 years) and no EGFRvIII expression (1.75 years) \(^{P = 0.547, \text{Fig. 3C}}\).

**Discussion**

Epidermal growth factor receptor variant III is known to promote angiogenesis through activation of c-myc and tumor growth through constitutive activation of the signal transducers and activators of transcription (STAT) and PI3K-Akt pathways. EGFR amplification is seen in about 40% of primary glioblastomas. EGFRvIII, a mutant EGF receptor, is overexpressed in 50–60% of EGFR-amplified glioblastomas, lacks the extracellular ligand-binding domain (exons 2–7 deletion) and is constitutively active. Some papers have reported the localization of EGFRvIII within gliomas to be more regional than EGFR. Physical interaction of EGFRvIII and EGFR, both paracrine and co-expressed within individual tumor cells, have also been elucidated. Recently, newer antibodies have been developed to detect EGFRvIII and the analysis of localization of EGFRvIII within gliomas has become possible.

In this study, we found EGFRvIII expression in cellular areas of the tumor in a subset of primary glioblastomas, but not at the invading zone. Xenograft models or cultured cell models have shown that tumor cells expressing EGFRvIII are less invasive than EGFRvIII-negative cells. In some reports, tumor cells in the peritumoral brain have been shown to have higher invasive potential. Taken together, the evidence suggests that EGFRvIII-expressing proliferating tumor cells may lose their expression before they become invasive.

The grow-or-go phenomenon, whereby cell motility and proliferation are mutually exclusive, is well documented in cancer. Hypoxia has been implicated in the switch from proliferation to invasion. Our results suggest that EGFRvIII may also be an important factor determining whether cancer cells “go-or-grow”.

It is known that in some glioblastomas, sharply delineated, round, GFAP-negative foci arise in the background of a more differentiated lesion. This phenomenon is thought to be induced by altered genetic expression, but the exact mechanism is unknown. A similar phenomenon was observed in case #3 (Table 2), where small, round, GFAP-negative tumor cells were found in the infiltrating area of a glioblastoma with a more differentiated core expressing EGFRvIII. These small cells lacked EGFRvIII expression.

Although EGFRvIII expression in tumors is known to be heterogeneous, previous studies have focused mainly on the tumor mass, and not the invading zone. Interestingly, a study by Eskilsson et al. found EGFRvIII expression near the border of...
Fig. 2  Representation of the features of case #4. (A) T₁-weighted magnetic resonance image with contrast enhancement (MRI-T₁CE) demonstrates a large tumor in the right frontal lobe. (B) A histological section of the resected brain. Klüver–Barrera stain. The cellular portion of the tumor indicated by square 1 shows a high nuclear concentration, whereas the peripheral portion indicated by square 2 exhibits relative myelin pallor. (C) A serial section stained with the EGFRvIII antibody (Absolute Antibody). EGFRvIII immunoreactivity is seen in the cellular portion of tumor. (D) A section stained with the EGFRvIII antibody (Millipore). (E) Higher-power magnification immunostained with the EGFRvIII antibody (Millipore) (F) Higher-power magnification views of the cellular portion (upper panels: corresponding to square 1 in B and C) and the peripheral portion (lower panels: corresponding to square 2 in B and C) of the tumor. In the central portion, highly cellular tumor cells show immunoreactivity for vimentin, EGFRvIII and GFAP, having multiple fine processes (arrows), whereas in the peripheral portion the infiltrating tumor cells show immunoreactivity for vimentin, but not for EGFRvIII or GFAP, and have a small, rounded morphology (arrowheads). Scale bars = 25 µm. (G) Double-labeling immunofluorescence views of the central portion (upper panels: corresponding to square 1 in B and C) and the peripheral portion (lower panels: corresponding to square 2 in B and C) of the tumor. Processes of large, well differentiated, GFAP-positive glial processes (arrowhead) extend to and surround less differentiated, EGFRvIII-positive glial cells in cellular areas of tumor (arrow), whereas in the peripheral portion the infiltrating tumor cells show no immunoreactivity for EGFRvIII or GFAP. Scale bars = 25 µm.
EGFRvIII Expression in Cellular Areas of Glioblastoma

The tumor in two cases of glioblastoma. This study demonstrated the location of EGFRvIII expression at cellular areas of tumor, whereas invading glioma cells lack such expression. A recent review article by Eskilsson et al. highlights the heterogeneity of EGFR in glioblastoma and crosstalk between wtEGFR, which enhances tumor cell invasion, and EGFRvIII, which promotes angiogenic tumor cell invasion, and also reviews the potential therapeutic implications to targeted therapies.

In two recurrent cases, EGFRvIII-positive cells were markedly decreased in one case and retained in the other (Figs. 3A and 3B). This observation is not consistent with a detailed study of somatic mutations in glioblastomas at the time of initial diagnosis, which found that EGFRvIII was expressed in the initial tumors but disappeared at relapse. A recent report has indicated that EGFRvIII can be eliminated from extrachromosomal DNA of tumor cells when treated with EGFR tyrosine kinase inhibitors, but reappears after drug removal. The exact mechanism in which EGFRvIII is eliminated and reemerges needs to be elucidated. These data suggest the importance of evaluating EGFRvIII status not only in the initial tumor, but also at relapse when using EGFRvIII-targeted treatments in the recurrent setting.

There are conflicting reports about the prognostic relevance of EGFRvIII. Some studies have suggested that patients with EGFRvIII-expressing glioblastomas have a good prognosis, whereas others have found no difference in survival. We were unable to demonstrate any such survival difference in this study. The extent of tumor resection has also been reported to affect survival, but in this study there was no difference in the extent of resection between the two groups. Given the central location of EGFRvIII-expressing cells within the tumor and the lack of EGFRvIII-expressing cells at the time of relapse, we can speculate that infiltrative EGFRvIII-negative cells may have a prognostic effect.

In conclusion, in a subset of primary glioblastomas, EGFRvIII is expressed in cellular areas of tumor where the tumor cells show features of astrocytic differentiation and GFAP positivity, whereas invading cells show no EGFRvIII expression. This finding suggests that EGFRvIII is involved in tumor...
proliferation, whereas invading glioma cells lose their expression of EGFRvIII.

Conflicts of Interest Disclosure

The authors declare that they have no conflict of interest. The authors who are members of the Japan Neurosurgical Society (JNS) have registered online Self-reported COI Disclosure Statement Forms through the website for JNS members.

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