Detection of *EGFR* Mutation Distribution and Transcriptional Variants in IDH-Wildtype High-Grade Gliomas Using a Next-Generation Sequencing Oncopanel

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Abstract

Purpose: To detect the epidermal growth factor receptor gene (*EGFR*) mutation profile and transcriptional variants in high-grade gliomas (HGGs), we sequenced *EGFR* and evaluated the *EGFR* splicing profile using a next-generation sequencing (NGS) oncopanel.

Methods: We analyzed 124 HGGs—10 grade III *IDH*-wildtype anaplastic astrocytomas (AAs) and 114 grade IV *IDH*-wildtype glioblastomas (GBMs).

Results: The *EGFR* mutations were observed in 6.0% of grade IV GBMs and in 33% of grade III AAs. Four cases harbored missense mutations in the *EGFR* kinase domain (L747A, S768I, V774M, and T790M). A total of 25% of the GBMs showed *EGFR* amplification. Moreover, 27% of the *EGFR* mutations occurred in the kinase domain. *EGFRvIII* positivity was detected in 8.0% of *EGFR*-amplified GBMs. We identified two other *EGFR* variants in GBM cases with deletions of exons 6–7 (Δ 6-7) (one case) and exons 2–14 (Δ 2-14) (two cases). Interestingly, in one case, the initial *EGFRvIII* mutation transformed into an *EGFR* Δ 2-14 mutation during recurrence. The frequency of *EGFR* alterations in our cohort was lower but the frequency of *EGFR* mutations in the kinase domain in our cohort was higher than that in The Cancer Genome Atlas and Memorial Sloan Kettering Cancer Center cohorts.

Conclusions: We suggested that the *EGFR* gene profiles of GBM differ among cohorts and identified rare *EGFR* variants with longitudinal and temporal transformations of *EGFRvIII*.

Introduction

The epidermal growth factor receptor gene (*EGFR*) is the most commonly amplified and overexpressed proto-oncogene in glioblastoma multiforme (GBM) and causes the most malignant subtype. *EGFR* amplification is detectable by next-generation sequencing (NGS) in approximately 40–50% of GBM cases [1–3], and 14.4–26% of GBMs harbor *EGFR* mutations [2, 3]. Recently, the Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) proposed a diagnostic entity of grade II–III isocitrate dehydrogenase (*IDH*)-wildtype astrocytoma that should behave similar to GBM. The molecular features defining this new tumor entity include telomerase reverse transcriptase promoter (*TERTp*) mutation, *EGFR* amplification, or a combination of whole chromosome 7 gain and whole chromosome 10 loss [4]. *EGFR* may serve as a diagnostic and prognostic biomarker. Indeed, *EGFR* has garnered interest as a drug target in GBM because of the high frequency of *EGFR* alterations in this disease. However, GBMs respond poorly to *EGFR* inhibitors [5, 6]. As *EGFR* mutations in GBM occur in the extracellular domain rather than the intracellular kinase domain, *EGFR* inhibitors that target the kinase domain are less effective [6].

Splicing variants of *EGFRvIII* (Δ 14-15 deletions), *EGFRvIII* (Δ 2-7 deletions), *EGFRvIII* (Δ 25-27 deletions), *EGFRvIII* (Δ 25-28 deletions), and *EGFR* (Δ 12-13 deletions) have been reported in GBMs [2, 3, 7, 8]. *EGFRvIII*, which is generated by the fusion of exons 1 and 8, is detectable in 50–60% of *EGFR*-amplified GBMs [9, 10]. *EGFRvIII* lacks the extracellular ligand-binding domain and constitutively activates the receptor tyrosine kinase domain that increases GBM cell proliferation, migration, and invasion [11, 12]. *EGFRvIII* may serve as a tumor-specific target for anti-*EGFRvIII* immunotherapy, including antibody-based approaches and vaccines, such as rindopepimut, which produces a survival signal when combined with bevacizumab in *EGFRvIII*-positive recurrent GBMs [13]. However, immunotherapy failed in a phase III clinical trial in newly diagnosed patients [14]. This may be due to the frequent loss of *EGFRvIII* expression with standard chemoradiotherapy.

To evaluate the *EGFR* mutational and splicing profile in *IDH*-wildtype high-grade gliomas (HGGs), we performed NGS analysis using a custom gene panel that we recently reported [15]. In this study, we collected a higher number of samples to clarify *EGFR* mutational distribution and developed a method to detect *EGFR* transcriptional variants.

Materials And Methods

Diffuse glioma samples

One hundred and twenty-four formalin-fixed paraffin-embedded (FFPE) tumor tissue samples were selected from the Central Nervous System Tumor Tissue Bank at Kagoshima University Hospital. The study was approved by the Institutional Review Board of Kagoshima University (approval number: 180104) and complied with the tenets of the Declaration of Helsinki. Informed consent was obtained from all patients. Resected tumors were fixed with phosphate-buffered 10% formalin within 24 h of sampling and routinely

processed for paraffin embedding, followed by sectioning for hematoxylin and eosin staining. All tumors were originally classified according to the World Health Organization classification of 2016. The tumor series consisted of 10 grade III *IDH*-wildtype anaplastic astrocytomas (AAs) and 114 grade IV *IDH*-wildtype GBMs. All tissues were histologically evaluated by board-certified pathologists (M.K. and A.T.) to ensure an estimated tumor cell content of 30% or more. In all patients, when analyzing copy number variations, we sequenced leukocyte DNA for comparison against matched tumor DNA.

DNA extraction and quantification

For DNA preparation from FFPE samples, we used the Maxwell 16 FFPE Tissue LEV DNA Purification kit (Promega) according to the manufacturer's instructions. Thereafter, the concentration of DNA was measured using a Qubit 3.0 Fluorometer dsDNA BR Assay kit (Life Technologies), and DNA quality was monitored using the QIAseq DNA QuantiMIZE kit (QIAGEN). The extracted DNA was diluted to a concentration of 5–10 ng/μL as a template, and polymerase chain reaction (PCR) was performed using the QIAseq DNA QuantiMIZE kit.

Next-generation sequencing

NGS was performed using an amplicon-based glioma-tailored gene panel as described previously [15]. Amplicon sequences were aligned to the human reference genome GRCh37 (hg19) in the target region of the sequence. Data were analyzed using the QIAGEN Web Portal service (<https://www.qiagen.com/>).

Detection of *EGFR* variants

In all patients, to analyze rearrangement events as significant targets of intragenic copy number breakpoints, we sequenced leukocyte DNA for comparison against matched tumor DNA. The *EGFR* variants were detected by reduced read counts obtained for *EGFR* sequences in tumor DNA relative to those in matched leukocyte DNA. In addition, as a validation method, the *EGFR* variants were detected by determining the copy number (CN) of each exon in *EGFR* and identifying exon sites with -2SD below in CN compared with the average CN of all exons.

Complementary DNA analyses of clinical specimens

The total RNA from FFPE samples of clinical specimens was extracted using the Maxwell 16 LEV RNA FFPE Purification kit (Promega) and converted into cDNA using the ReverTra Ace qPCR RT Kit & Master Mix (TOYOBO Inc., Osaka, Japan). The cDNA fragments around the targeted site were amplified by PCR using the KOD One PCR Master Mix (TOYOBO Inc.) using the primers listed in Supplementary Table S1. The PCR products were analyzed by electrophoresis on 2% agarose gels. The PCR fragments were purified using the Exo-CIP Rapid PCR Cleanup kit (New England Biolabs Inc., Ipswich, MA) and analyzed by GENEWIZ Japan Corp.

Immunohistochemistry

Surgical specimens were fixed within 10 min of excision in 10% neutral buffered formaldehyde for 24 h, embedded in paraffin, cut into 3-μm-thick sections, and mounted on glass slides coated with poly-L-lysine. Subsequently, the sections were probed with 1:25 anti-EGFR (mouse monoclonal antibody, clone EGFR.113; Leica Biosystems) and 1:200 anti-EGFRv3 (rabbit monoclonal antibody, cat#64952; Cell Signaling Technology). The sections were then stained with diaminobenzidine tetrahydrochloride and hematoxylin.

TCGA and MSKCC data analyses

We retrieved the molecular characteristics of the GBM cohort from previous publications. After excluding *H3F3A*, *IDH1/2*, and *BRAF* V600E-mutant cases, we analyzed 343 cases from The Cancer Genome Atlas (TCGA) cohort and 456 cases from the Memorial Sloan Kettering Cancer Center (MSKCC) cohort [16]. All cases were conclusively diagnosed as *IDH*-wildtype GBM using the cBioPortal for Cancer Genomics (<https://cbioportal.org>).

Definition of actionable alterations

Actionable gene alterations were predicted to confer sensitivity to either an approved targeted agent or an experimental targeted agent currently under clinical trials. The JAX Clinical Knowledgebase (JAX-CKB) (<https://ckb.jax.org/>) was used to identify clinically actionable variants.

Data analysis

We used OncoPrinter (cbioportal.org/oncoprinter) and MutationMapper (cbioportal.org/mutation_mapper), which are tools in the cBioPortal for Cancer Genomics, to visualize and analyze our data [17,18]. Statistical analyses were performed using JMP Pro v13 software (SAS Institute, Cary, NC). Groups were compared using the χ^2 test. Differences were considered significant at $p < 0.05$.

Results

EGFR alterations in HGGs

We analyzed 124 HGGs—10 *IDH*-wildtype AAs and 114 *IDH*-wildtype GBMs. EGFR alterations were detected in 25% of *IDH*-wildtype GBMs (Fig. 1a) and 60% of *IDH*-wildtype AAs (Fig. 1a). The EGFR mutation frequency in *IDH*-wildtype AAs was higher than that in *IDH*-wildtype GBMs (50% and 6.1%, respectively; $p < 0.001$) (Table 1). Three cases had 2 EGFR point mutations and one case had 3 EGFR point mutations (Table 1). We also identified mutations in the EGFR extracellular and intracellular domains in 64.7% (11/17 mutation sites) and 35.3% (6/17 mutation sites) HGGs, respectively (Fig. 1b). Four cases harbored missense mutations in the EGFR kinase domain (encoding L747A, S768I, V774M, and T790M) (Table 1). In GBMs, 27% (3/11 mutation sites) EGFR mutations were kinase domain mutations (Fig. 1b), and 18.2% (2/11 mutation sites) EGFR mutations were druggable and registered in the JAX-CKB database (Fig. 1c). Furthermore, 20% patients with *IDH*-wildtype AA and 1.8% of patients with *IDH*-wildtype GBM harbored druggable mutations registered in the JAX-CKB database (Table 1). Additionally, 50% patients with *IDH*-wildtype AA and 21.9% patients with *IDH*-wildtype GBM had EGFR amplifications.

EGFR variants in HGGs

The EGFRv3 (Δ 2-7) variant was detected by NGS in three (1 AA and 2 GBMs) of the 30 tumors with EGFR amplification (Table 2). All EGFRv3-positive cases were verified by immunohistochemistry and reverse transcription-PCR (RT-PCR) (Fig. 2a, d). EGFRv3 was detected in two of the 25 EGFR-amplified GBMs (8.0%). In addition, we identified two other EGFR variants in GBM cases, namely deletions of exons 6–7 (Δ 6-7) and exons 2–14 (Δ 2-14). EGFR Δ 6-7 was detected in 1 case, and EGFR Δ 2-14 was detected in 2 cases (Table 2). Both EGFR Δ 6-7 and EGFR Δ 2-14 were verified by RT-PCR (Fig. 2b, c, d). All EGFR variants showed EGFR amplification (Table 2).

Longitudinal and temporal transformation of EGFRv3

As an example, a 57-year-old woman with complaints of seizures underwent resection for a lesion in the left temporal lobe with high FLAIR signals observed by magnetic resonance imaging and high methionine accumulation observed by positron emission tomography. The pathological diagnosis was *IDH*-wildtype AA with an EGFRv3 (Δ 2-7) variant (Fig. 3a). Two years later, a tumor recurred in the same area, and the patient underwent another resection. The pathological diagnosis was *IDH*-wildtype GBM with EGFR exon 2–14 deletion (Δ 2-14) without EGFRv3 (Δ 2-7) (Fig. 3b). In other words, the initial EGFRv3 (Δ 2-7) mutation transformed into EGFR exon 2–14 deletion (Δ 2-14), with extensive deletion of exons 8–14 at the time of recurrence.

Frequencies of genetic alterations differed among the cohorts

The frequency of EGFR alterations in our cohort (Fig. 1a) was lower than that in TCGA cohort (25% and 57%, respectively; $p < 0.001$) and MSKCC cohort (25% and 44%, respectively; $p < 0.001$). However, the frequency of EGFR mutations in the kinase domain in our cohort (Fig. 1a) was higher than that in TCGA cohort (27% and 5.9%, respectively; $p = 0.0401$) and MSKCC cohort (27% and 4.1%, respectively; $p = 0.0134$) (Supplementary Fig. 1).

Discussion

In this study, we collected higher number of samples than in our previous study [15]. We found that the frequency of EGFR mutations was low but the frequency of EGFR mutations in the kinase domain was high in wildtype-GBMs. Moreover, we developed a method to detect EGFR transcriptional variants; we found a novel EGFR transcriptional variant and identified a case with a rare EGFR variant with longitudinal and temporal transformations of EGFRv3.

EGFR amplification is detectable in approximately 40–50% of all GBMs [1–3]. The frequency of *EGFR* alterations in our cohort was lower than that in TCGA and MSKCC cohorts. However, a study from Japan reported that 25.5–33.1% of GBMs have *EGFR* amplifications [19, 20], which is consistent with our findings. Recent reports indicate that 14.4–26% GBMs harbor *EGFR* mutations [2, 21]; however, the *EGFR* mutation frequency in GBMs in our cohort (6.0%) was considerably lower than that in the previous study cohort. These results suggest that *EGFR* alterations may be less frequent in Japan than in other countries. Moreover, in our study, *EGFR*^{A289D/T/V} was the most common missense mutation, and two cases harbored *EGFR*^{T790M} missense mutations in the *EGFR* kinase domain. Previous studies have shown that approximately 4% *EGFR* mutations in GBM have mutations in the kinase domain [2, 21]; however, in our study, 27% *EGFR* mutations were in the kinase domain in GBMs, and this is higher than that reported previously.

Two cases (1 GBM and 1 AA) harbored *EGFR*^{T790M} missense mutations in the kinase domain. *EGFR*^{T790M}, which is commonly observed in lung cancer [22], is a very rare mutation in glioma, with only one case of GBM reported previously [23]. Osimertinib is an oral, third-generation tyrosine kinase inhibitor (TKI) that irreversibly inhibits *EGFR* and was developed specifically to target the *EGFR*^{T790M}-resistant mutation in *EGFR*-mutated non-small-cell lung cancer [22, 24, 25]. Makhin et al. reported a GBM case with two *EGFR* point mutations (C628F and A289V) that responded well to osimertinib. Thus, glioma cases with mutations in the *EGFR* kinase domain may benefit from *EGFR* TKIs [26]. In our study, a high *EGFR* amplification was identified in 5/10 (50%) patients with *IDH*-wildtype AA, consistent with the findings of a previous study [27]. In patients with *IDH*-wildtype AA, 33% *EGFR* mutations (2/6 mutation sites) had T790M and S768I mutations in the kinase domain. However, in *IDH*-wildtype AA, the distribution of mutations within the *EGFR* coding sequence has not been reported.

EGFRv3 is the most common *EGFR* splice variant. *EGFRv3* activates multiple downstream signaling pathways and exhibits high tumorigenic potential [8, 11, 12]. Recent reports indicate that 50–60% *EGFR*-amplified GBMs harbor *EGFRv3* variants [9, 10]. *EGFRv3* positivity was detected in 2 of the 25 *EGFR*-amplified glioblastomas (8.0%) in our study, suggesting that *EGFRv3* positivity may have been less frequent in our study than in previous studies. These discrepancies may be due to ethnic differences in patient cohorts or differences in analysis methods; we used NGS and not immunohistochemistry or RT-PCR. A comparative study found that RT-PCR was more sensitive and specific than immunostaining using two different *EGFRv3*-specific antibodies [28]. Another study showed that the sensitivity of NGS-based *EGFRv3* detection is lower than that of immunohistochemistry or RT-PCR, reflecting that *EGFRv3* may be restricted to small subclones of glioma cells, which may not lead to a detectable reduction in exon 2–7 gene dosage [29].

In this study, we identified one uncharacterized *EGFR* variant with deletions of exons 6–7 (Δ e 6-7). Deletions of exons 2–14 (Δ e 2-14) constitute a very rare variant in glioma, with only one case of GBM having been reported previously [30]. In one case, the initial *EGFRv3* (Δ e 2-7) mutation transformed into a *EGFR* exon 2-14 deletion (Δ e 2-14) at the time of recurrence. Recent reports indicate that 16–59% of GBMs that were initially *EGFRv3* positive, lost *EGFRv3* at recurrence [9, 10, 14]. Some reports suggest that the frequency of *EGFRv3* loss at recurrence is altered by the treatment received [31], whereas others suggest that it is not dependent on the treatment received [14]. In addition, it has been reported that the expression of *EGFRv3* is a result of epigenetic regulation [32], but the mechanism by which *EGFRv3* is lost at recurrence is unknown. Based on our findings, it is possible that the initial *EGFRv3* mutation is transformed to other variants during recurrence. The functional significance of the novel *EGFR* variants needs to be analyzed in further studies.

In conclusion, we report the distribution of mutations within the *EGFR* coding sequence and two novel *EGFR* variants, one of which showed longitudinal and temporal transformation of *EGFRv3*. In addition, we showed that the frequency of driver gene alterations in GBMs differs across cohorts. Thus, to implement personalized medicine, it is necessary to accurately assess the genetic profile of each cohort.

Declarations

Funding

The authors received no financial support related to this study.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material

All data used and analysed in the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

Conception and design: AT, KY; development of methodology: TA, TH, SY, TT; acquisition of data: NH, HY, HU, MK, RM, SW, SF; analysis and interpretation of data: NH, TA, TH, SY; manuscript writing, review, and/or revision: NH, RH, AT, KY; administrative, technical, or material support (e.g., reporting or organizing data and constructing databases): NH, TT, HY, HU, TA, TH, SY; study supervision: RH, AT, KY.

Ethics approval

The study was approved by the Institutional Review Board of Kagoshima University (approval number: 180104) and complied with the tenets of the Declaration of Helsinki.

Consent to participate

Informed consent was obtained from all patients

Consent for publication

All authors agreed to the publication of the manuscript.

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Tables

Table 1. High grade glioma cases with EGFR gene alterations

Research ID	Pathology	Sex	Age	SNV	CNV		Vaf (%)	impact	mutation effect	evidence type
2	GBM	male	81	p.A289D	19.43	amplification	80	missense	gain of function	-
8	GBM	female	70	p.S229C	1.76	intact	28.9	missense	gain of function-predicted	-
				p.G588D			43.4	missense	unknown	-
83	GBM	male	63	p.V774M	29.40	amplification	75.4	missense	unknown	-
				p.T790M			80.5	missense	gain of function	druggable mutation
85	GBM	male	78	p.L747A	2.48	intact	19.3	missense	unknown	-
93	GBM	female	65	p.R108K	20.79	amplification	33.2	missense	gain of function	-
				p.A289V			24.17	missense	gain of function	druggable mutation
				p.D984Y			83.37	missense	unknown	-
96	GBM	male	51	p.S442I	2.00	intact	19.3	missense	unknown	-
97	GBM	male	73	p.F254I	1.98	intact	29.39	missense	unknown	-
19	AA	male	49	p.R108K	10.49	amplification	70	missense	gain of function	-
52	AA	female	71	p.A289D	104.10	amplification	12	missense	gain of function	-
53	AA	male	61	p.A289T	35.77	amplification	42.5	missense	gain of function	-
59	AA	male	67	p.G598V	2.57	intact	23.6	missense	gain of function	-
				p.S768I			19.2	missense	gain of function	druggable mutation
90	AA	male	39	p.T790M	5.52	amplification	28.1	missense	gain of function	druggable mutation

Table 2. Repertoire of EGFR variant detecting in high grade glioma cases

Research ID	Pathology	Sex	Age	Type of variants	CNV	
15	AA	female	57	EGFRv	42.1	amplification
16	GBM	female	60	EGFR exon 2-14 del	25.5	amplification
21	GBM	female	82	EGFR exon 2-14 del	58.9	amplification
28	GBM	male	62	EGFRv	52.8	amplification
49	GBM	male	64	EGFRv	169.4	amplification
112	GBM	male	77	EGFR exon 6-7 del*	165.1	amplification
113	GBM	male	72	EGFRv	82.2	amplification

*:novel EGFR variant

Figures

Figure 1

Somatic alterations of EGFR. (a) EGFR mutations and copy number alterations were generated and visualized using OncoPrinter included in the cBioPortal for Cancer Genomics software suite. (b) EGFR protein domain structure with somatic mutations summarized from 114 IDH-wildtype GBMs and 10 IDH-wildtype AAs. (c) Sunburst chart showing the EGFR mutation spectrum in IDH-wildtype GBMs and IDH-wildtype AAs. Percentages were calculated from the total EGFR mutation sites

Figure 2

Detection of EGFR variants. (a) EGFRvIII was indicated by reduced read counts for exons 2–7 in tumor DNA relative to those for exons 2–7 in matched leukocyte DNA (upper panel) and was detected based on reduced CN of exons 2–7 compared with the average CN of all exons (lower left panel). EGFRvIII was validated using RT-PCR (lower right panel). (b) EGFR exon 2–14 deletion was indicated by reduced read counts for exons 2–14 in tumor DNA relative to those for exons 2–14 in matched leukocyte DNA (upper panel). It was detected based on reduced CN of exons 2–14 compared with the average CN of all exons (lower left panel). EGFR exon 2–14 deletion was validated using RT-PCR (lower right panel). (c) EGFR exon 6–7 deletion was indicated by reduced read counts for exons 6–7 in tumor DNA relative to those for exons 6–7 in matched leukocyte DNA (upper panel). It was detected based on reduced CN of exons 6–7 compared with the average CN of all exons (lower left panel). EGFR exon 6–7 deletion was validated using RT-PCR (lower right panel). (d) Structures of EGFR and its splicing variants. EGFRvIII (exon 2-7 deletion), exon 6-7 deletion, and exon 2-14 deletion are shown

Figure 3

Longitudinal transformation of EGFRvIII. (a) Upper: Enhanced T1WI, FLAIR, and methionine PET in an initial GBM case with IDH-wildtype AA. Middle: EGFRvIII detected by NGS and RT-PCR. Lower: Hematoxylin, eosin, and immunostaining of EGFR wildtype and EGFRvIII. (b) Upper: FLAIR and methionine PET in a recurrent GBM case with IDH-wildtype GBM. Middle: EGFRvIII detected by NGS and RT-PCR. Lower: Hematoxylin, eosin, and immunostaining for EGFR wildtype and EGFRvIII

Supplementary Files

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