Pathol. Oncol. Res. (2009) 15:225–229
DOI 10.1007/s12253-008-9082-4

ORIGINAL PAPER

Analysis of EGFR Gene Amplification, Protein Over-expression and Tyrosine Kinase Domain Mutation in Recurrent Glioblastoma

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Received: 27 May 2008 / Accepted: 7 July 2008 / Published online: 28 August 2008

Abstract Gefitinib and erlotinib are both selective EGFR tyrosine kinase inhibitors (EGFR-TKIs) that have produced responses in a small subgroup of lung cancer patients. The strongest evidence for a role of EGFR in the biology of glioblastoma stems from clinical trials in which 15–20% of recurrent glioblastoma patients experienced significant tumour regression in response to these small-molecule EGFR kinase inhibitors. We examined the protein-kinase domain of the EGFR gene, EGFR protein expression and EGFR gene amplification in 20 cases of recurrent GBMs. EGFR protein over-expression was found in 65% of cases. EGFR protein over-expression was associated with EGFR gene amplification in 35% of cases, and with high polysomy in 15% of cases. No mutations were found in the TK domain of the EGFR gene. Our results confirm that mutations in the kinase domain are absent in recurrent GBM, and this might be a preponderant factor in the lack of major clinical responses of TKIs in GBM, recent studies have suggested that responsiveness to EGFR kinase inhibitors was strongly associated with coexpression of EGFRvIII and PTEN. Further prospective validation of EGFRvIII and PTEN as predictors of the clinical response to EGFR kinase inhibitors in recurrent GBM is strongly anticipated.

Keywords Glioblastoma multiforme · EGFR · Kinase inhibitor treatment

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Introduction

Glioblastoma multiforme (GBM) is the commonest primary malignant tumour of the central nervous system in adults. Despite tangible progress, results remain disappointing, and median survival is 10–12 months; however, there is considerable variability in the prognosis among the patients diagnosed with GBM. Prognostic indicators include age, and extent of surgical resection. [1, 2] The most frequent genetic alteration associated with GBM is amplification of the epidermal growth factor receptor (EGFR) gene, which results in over-expression of the EGFR, a transmembrane tyrosine kinase receptor [3].

Epidermal growth factor receptor (EGFR) is commonly over-expressed and mutated in human malignancies and often associated with aggressive behaviour [4]. Before the recent discovery of the somatic mutations in the EGFR kinase domain in non-small cell lung cancers (NSCLC), deletions of the extracellular domain were considered the most frequent EGFR mutation in different tumor types [5, 6]. The commonest mutated receptor is the variant III EGFR deletion mutant (EGFRvIII, delta 801EGFR, del2–7

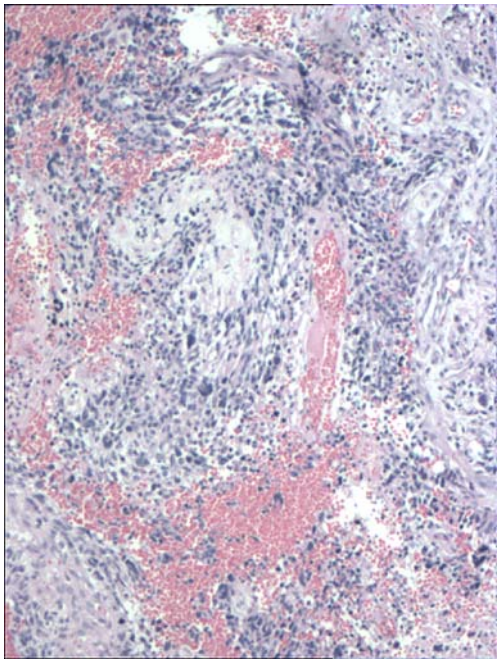

Fig. 1 Recurrent glioblastoma multiforme (hematoxylin–eosin). The tumour shows pleomorphism and necrosis

EGFR), containing an in-frame deletion of exons 2–7 (801 bp) from the extracellular domain, initially characterized at the genomic level in GBM. The in-frame deletion of exons 2–7 has an activating effect on the receptor, giving cells expressing these abnormal receptors a proliferative advantage [7, 8].

Gefitinib and erlotinib are both selective EGFR tyrosine kinase inhibitors (EGFR-TKIs) that have produced responses in a small subgroup of lung cancer patients, with a statistically significant improvement in survival compared with best supportive care in the case of erlotinib. Patient response to EGFR-targeted anticancer therapy can be directly correlated with an underlying somatic-activating mutation in the tumour EGFR gene. The commonest EGFR mutations include the deletion of four conserved amino-acid residues (LREA) in exon 19 and a point mutation, L858R in exon 21 [9, 10].

The strongest evidence for a role of EGFR in the biology of glioblastoma stems from clinical trials in which 15–20% of recurrent glioblastoma patients experienced significant tumour regression in response to small-molecule EGFR kinase inhibitors. Mutations in the EGFR kinase domain are unlikely to determine the sensitivity of glioblastomas to EGFR kinase inhibitors, as shown by numerous papers [11–14].

In these studies, EGFR status was mostly determined by examining of primary tumour samples rather than from recurrent tumours. However, the potential changes in the tumour's EGFR status due to postoperative chemotherapy or radiotherapy effects have not been addressed. In order to understand the effect of adjuvant therapy on the EGFR gene in relation to small-molecule EGFR kinase inhibitors we concluded to examine the protein-kinase domain of the EGFR gene, EGFR protein expression and EGFR gene amplification in a small series of recurrent GBMs.

Materials and Methods

Patient Selection

Twenty selected cases of recurrent GBM were collected. All patients had recurrences after receiving primary surgery and at least radiotherapy. The original tissue blocks were retrieved and haematoxylin–eosin stained slides were re-examined from each case to confirm the diagnosis (Fig. 1).

Immunohistochemistry

Immunohistochemistry was performed with a monoclonal antibody against the EGFR protein (clone 2–18C9; PharmDx Kit, DAKO, Glostrup, Denmark) according to the manufacturer's instructions. Membranous staining in ≥1% of tumour cells was considered positive, according to the EGFR PharmDx scoring guidelines. Appropriate positive and negative controls were used (Fig. 2).

Fluorescence In Situ Hybridization

Four-micrometer sections were treated with the Paraffin Pretreatment Reagent Kit II before hybridization (Vysis, IL,

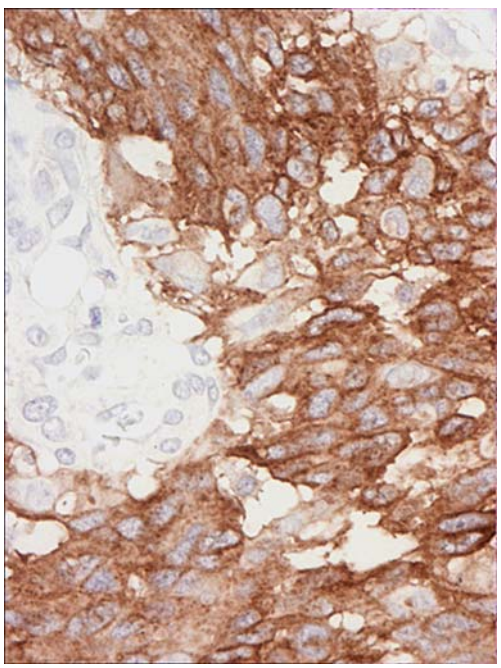

Fig. 2 EGFR expression in recurrent GBM (immunohistochemical reaction). The tumour cells strongly express the EGFR protein

USA) according the manufacturer's instructions. FISH was performed with a Spectrum Orange-labeled EGFR gene probe in combination with a Spectrum Green-labeled centromeric probe for chromosome 7 as a reference (Vysis). Slides were counterstained with 125 ng/ml 4′,6-diamino-2-phenylindole (Vysis) in antifade solution. The green and red signals were counted in 60 tumour nuclei per tissue sample.

Patients were classified into six groups with ascending EGFR gene copy numbers. Disomy was defined as ≤2 copies in >90% of cells, low trisomy as ≤2 copies in ≥40% of cells, 3 copies in 10–40% of the cells, ≥4 copies in <10% of cells, high trisomy as ≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells, low polysomy as ≥4 copies in 10–40% of cells, high polysomy as ≥4 copies in ≥40% of cells, and gene amplification as the presence of EGFR gene clusters or a ratio of EGFR gene to chromosome 7 of ≥2.

DNA Isolation

Using DNA-free conditions, formalin-fixed, paraffin-wax-embedded samples were cut into 10 μm sections on a microtome with a disposable blade. Deparaffinised tissue was resuspended in a proteinase K-containing buffer and incubated at 60°C for 16 h until the tissue was completely solubilised. DNA was purified with EZ1 Biorobot with EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany).

Nested PCR and DNA Sequencing

Eight pairs of primers targeting exons 18, 19, 20 and 21 of EGFR were used as described previously for nested polymerase chain reaction (nPCR).

The first PCR contained 100 ng of template DNA, 300 nM of the forward and reverse primers, 5 μl Expand High Fidelity PCR (Roche, Basel, Switzerland) master mix, 2.6 U Expand High Fidelity enzyme mix (Roche) and 200 μM of each dNTP in 50 μl. Samples amplified by the first round of PCR were subjected to a second round of nested PCR. The second PCR contained 3 μl from the first PCR and 300 nM of the forward and reverse primers, 5 μl Expand High Fidelity PCR (Roche) master mix, 2.6U Expand High Fidelity enzyme mix (Roche) and 200 μM of each dNTP in 50 μl.

PCR cycling parameters were one cycle of denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s (denaturation), 56°C for 30 s (annealing) and 72°C for 1 min (extension). PCR products amplified after the second round of PCR were loaded into a 2% agarose gel and electrophoresed to check the presence a single band of DNA, and PCR products were purified with High Pure PCR Product Purification Kit (Roche). The purified DNA was sequenced using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using 20 ng PCR product, 3.2 pmol forward or reverse primer and 8 μl Terminator Ready Reaction Mix in a total volume of 20 μl. The sequencing reaction was performed according to the manufacturer's description. After purifying the sequencing reaction with DyeEx 2.0 Kit (Qiagen, Hilden, Germany) the electrophoresis and data analysis were performed on an ABI 310 Genetic Analyzer (Applied Biosystems).

Results

EGFR Gene Amplification and Protein Over-expression

EGFR protein over-expression was found in 13 cases (65%). EGFR protein over-expression was associated with EGFR gene amplification in seven cases, and with high polysomy in three cases. The remaining three cases showed neither amplification nor high polysomy.

Patients were subdivided into FISH+ (amplification or high polysomy) and FISH− (disomy, low trisomy, high trisomy, and low polysomy) groups. Ten out of 20 cases were FISH+(50%). EGFR amplification was found in seven cases (35%), and high polysomy was seen in three tumours (15%; Fig. 3).

EGFR Sequence Analysis

The exons 18–21 of the EGFR gene showed no mutation. All cases revealed preserved DNA sequence in the exons examined.

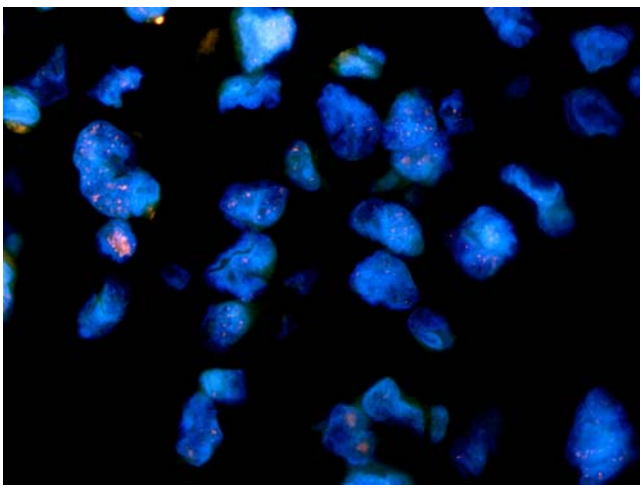

Fig. 3 EGFR amplification in recurrent GBM (FISH reaction). The *red* signals show the EGFR locus, which demonstrates amplification

Discussion

EGFR gene is amplified or EGFR protein is over-expressed in up to 60% of glioblastomas. Such alterations are a hallmark of the 'primary' glioblastoma as opposed to glioblastomas secondarily arising from low-grade gliomas in which these abnormalities are rare [3]. Mutations of the EGFR gene are frequent in glioblastomas and may be observed in up to 50% to 70% of EGFR over-expressing tumours. However, the vast majority of mutations affects the extracellular domain and involve a large deletion in exons 2 to 7. The resulting mutant receptor, termed EGFRvIII, has a ligand independent kinase activity and is observed in 60% to 70% of EGFR over-expressing glioblastomas [15]. However, it is important to mention that the PharmDX kit used in this study does not distinguish the mutant and wild forms of the EGFR receptor.

Most current research efforts are concentrated on EGFR TKIs. In 2004, two independent studies discovered an underlying association between mutations in the EGFR TK domain and gefitinib-responsive NSCLC [5, 6]. These EGFR kinase mutations enhance ligand-dependent activation of EGFR, while simultaneously increasing sensitivity to TKIs. TKI drugs (gefitinib, erlotinib) act by competing ATP for binding to the kinase pocket of the receptor, thus blocking receptor activation.

Among patients with glioblastoma only a small subgroup seems to benefit from the EGFR kinase inhibitors. The EGFR gene is commonly amplified in glioblastoma, but this abnormality also does not correlate with responsiveness to EGFR kinase inhibitors. The infrequency of mutations in the EGFR kinase domain in glioblastomas suggests that such EGFR mutations cannot account for responsiveness to EGFR kinase inhibitors [8, 11–13].

However, a number of conceptual issues may confound the correlative analysis of EGFR mutations and response. First, most retrospective studies include tumour specimens collected at initial diagnosis, whereas TKI therapy may have been administered after multiple courses of chemotherapy. As such, additional mutations leading to TKI resistance may have arisen in the interim, and may account for some mutation-positive unresponsive cases. On the contrary, additional activating mutations may occur later, following the initial treatment and the original genetic examination.

De Pas et al. suggested a very good overall agreement of EGFR status before and after systemic chemotherapy in EGFR-positive non small cell lung cancer, and their observation explained that a rebiopsy in an EGFR-positive patient after chemotherapy to reassess EGFR status is not necessary. However, their study also suggested that induction chemotherapy can induce EGFR expression in occasional patients with EGFR-negative tumors. The observation of this switch is consistent with the hypothesis that the EGFR ligand could be used as a survival factor to rescue from chemotherapy-induced damage [16].

In our study, EGFR protein over-expression was found in 13 cases (65%). EGFR protein over-expression was associated with EGFR gene amplification in seven cases, and with high polysomy in three cases. No mutations were found in the TK domain of the EGFR gene. The results demonstrate that while EGFR over-expression and gene amplification may still be present after chemotherapy, mutations affecting the TK domain of the EGFR cannot be found in recurrent glioblastoma. Nevertheless, the number of cases included in our study was relatively small; larger series of cases may reveal mutations of the TK domain.

However, our results confirm that mutations in the kinase domain are absent in recurrent GBM, and this might be a preponderant factor in the lack of major clinical responses of TKIs in GBM, recent studies have suggested that responsiveness to EGFR kinase inhibitors was strongly associated with coexpression of EGFRvIII and PTEN. Further prospective validation of EGFRvIII and PTEN as predictors of the clinical response to EGFR kinase inhibitors in recurrent GBM is strongly anticipated.

Ongoing clinical trials tend to show response with a certain frequency to kinase inhibitors in glioblastomas, but according to our results it is not the mutation analysis of the kinase domain that defines potentially responsive recurrent glioblastomas. Besides EGFRvIII and PTEN coexpression, different mutations affecting molecules of the downstream signal transduction pathway could also be considered in the assessment, with special regard to k-ras. However, in contrast to non-small cell lung cancer and colorectal carcinoma, k-ras mutations are extremely rare in GBM. The exact biology and the optimal therapeutic approach to glioblastoma multiforme is yet to be determined.

References

1. Bruner JM (1994) Neuropathology of malignant gliomas. Semin Oncol 21(2):126–138
2. Gilbert MR, Loghin M (2005) The treatment of malignant gliomas. Curr Treat Options Neurol 7(4):293–303
3. Rich JN, Hans C, Jones B et al. (2005) Gene expression profiling and genetic markers in glioblastoma survival. Cancer Res. 65(10): 4051–4058
4. Nicholson RI, Gee JM, Harper ME (2001) EGFR and cancer prognosis. Eur J Cancer 37(suppl 4):S9–S15
5. Lynch TJ, Bell DW, SordellaR et al. (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness

of non-small-cell lung cancer to gefitinib. N Engl J Med 350:2129–2139
6. Paez JG, Janne PA, Lee JC et al. (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 304:1497–1500
7. Nathoo N, Goldlust S, Vogelbaum MA (2004) Epidermal growth factor receptor antagonists: novel therapy for the treatment of high-grade gliomas. Neurosurgery 54:1480–1488
8. Barber TD, Vogelstein B, Kinzler KW et al. (2004) Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 351:2883–2883
9. Kris MG, Natale RB, Herbst RS et al. (2003) Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 290:2149–2158
10. Fukuoka M, Yano S, Giaccone G et al. (2003) Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 21:2237–2246
11. Mellinghoff IK, Wang MY, Vivanco I et al. (2005) Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 353:2012–2024
12. Rich JN, Reardon DA, Peery T et al. (2004) Phase II trial of gefitinib in recurrent glioblastoma. J Clin Oncol 22:133–142
13. Li B, Chang CM, Yuan M et al. (2003) Resistance to small molecule inhibitors of epidermal growth factor receptor in malignant gliomas. Cancer Res 63:7443–7450
14. Rich JN, Rasheed BK, Yan H (2004) EGFR mutations and sensitivity to gefitinib. N Engl J Med 351:1260–1261
15. Aldape KD, Ballman K, Furth A et al. (2004) Immunohistochemical detection of EGFRvIII in high malignancy grade astrocytomas and evaluation of prognostic significance. J Neuropathol Exp Neurol 63:700–707
16. De Pas T, Pelosi P, de Braud F et al. (2004) Modulation of epidermal growth factor receptor status by chemotherapy in patients with locally advanced non-small-cell lung cancer is rare. J Clin Oncol 22:4966–4970