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Clinical significance of *EGFR* amplification and the aberrant *EGFRvIII* transcript in conventionally treated astrocytic gliomas

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Abstract

The aim of this study was to evaluate the clinical value of assessing *EGFR* amplification, and the common 5' rearrangement of *EGFR* resulting in the *EGFRvIII* transcript in astrocytic gliomas. The data from 221 tumours was correlated with patient survival. The majority of previous studies evaluated amplification alone and have provided contradictory results. Amplification was analyzed by densitometry of Southern blots or quantitative PCR. *EGFR* transcripts were examined by RT-PCR and subsequent sequencing. An RNase protection assay was carried out on a subgroup to confirm the PCR results. Amplification of *EGFR* was found in 41% (65/160) of glioblastomas (GB), and 10% (4/41) of anaplastic astrocytomas (AA). The *EGFRvIII* rearrangement was identified in 54% (35/65) of GB and 75% (3/4) of AA with amplification as well as in 8% (8/95) of GB and 5% (2/37) of AA without amplification (confirmed by RNase protection assay). There were no abnormalities of the *EGFR* or its transcript in astrocytomas malignancy grade II (AII). We found no significant association between *EGFR* amplification or rearrangement and age or survival in the 160 GB patients. We noted a tendency towards decreased survival in the 41 patients with AA with any *EGFR* abnormality. This was most marked in the 5 cases with the *EGFRvIII* transcript ($p=0.069$) but these were significantly older than those without ($p=0.023$). No abnormalities of *EGFR* were identified in AII patients. We conclude that neither *EGFR* amplification nor the presence of the *EGFRvIII* transcript predict patient outcome in conventionally treated GB. In AA however, although uncommon, *EGFR* aberrations appear to be associated with shorter survival.

Keywords

EGFRvIII; survival; transcript; Glioblastoma

Introduction

The normal epidermal growth factor receptor (*EGFR*) gene on 7p codes for a 170 kDa transmembrane glycoprotein expressed in a variety of normal and malignant cells. It is composed of an N-terminal extra-cellular ligand-binding domain, a hydrophobic transmembrane region, a cytoplasmic part with intrinsic tyrosine kinase activity and a carboxy-terminal region with tyrosine residues and regulatory motifs (1). At least 6 ligands for the EGFR are known (2). When activated, wild-type EGFR forms homodimers or

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heterodimers (with other members of the erbB receptor family), triggering a number of signalling cascades via the Ras-mitogen-activated protein kinase (Ras-MAPK) pathway and the phosphatidyl-inositol 3' kinase-protein kinase B (PI3K-Akt) pathway (1).

EGFR signalling is abnormal in a variety of tumour types, both of epithelial and neuroepithelial origin. In addition to amplification and/or overexpression, the amplified *EGFR* gene is frequently rearranged in Glioblastomas (GB) (3-6). Numerous different rearrangements have been reported, the commonest being the 140–155 kDa EGFRvIII - a well-characterized and constitutively active variant with an in-frame deletion involving exons 2–7 (6-8).

Among astrocytic tumours aberrations of *EGFR* are most common in GBs (WHO grade IV) and particularly so in “primary” (or “*de novo*”) GBs (9-11). A small fraction (up to 18%) of Anaplastic Astrocytomas (AA; WHO grade III) also have *EGFR* amplification, while amplification in WHO grade II Astrocytomas (AII) has only been reported in single cases (12, 13). GBs are the most frequent and most malignant human primary brain tumour in adults (14). In GB *EGFR* is amplified in 30-40% (15, 16) and rearranged in up to approximately 20% of GB, generally in combination with amplification (3, 17). Attempts to correlate *EGFR* amplification analysis alone with outcome in GB and/or AA patients treated with conventional therapy (i.e. gross total surgical resection followed by external beam radiation therapy) have to-date provided inconclusive or contradictory results (9, 10, 12, 13, 18-20).

Here we have analyzed the *EGFR* gene for both amplification and the common *EGFRvIII* rearrangement in a series of 221 astrocytic gliomas. The molecular findings have been correlated with survival to assess whether knowledge of these factors currently provides information of any value for clinicians. We show that with conventional therapy analysis the *EGFR* gene status, and its transcripts are of little clinical value in cases of bona fide glioblastomas. However, in anaplastic astrocytomas the presence of *EGFR* amplification and/or rearrangement may indicate shorter survival. However, the on-going development of novel therapies targeting tyrosine kinase receptors or specifically EGFRvIII may make this analysis essential in selecting patients for such treatments.

Materials and Methods

Tumour Samples and DNA/RNA Extraction

A total of 221 patients with “primary”, non-recurrent astrocytic tumours operated at the Karolinska Hospital, Stockholm, or Sahlgrenska University Hospital, Gothenburg, between 1988 and 1997 were included in the study. In 10 cases tumour from 2 operations was available. All tumours were extensively sampled for histology and re-assessed according to the histological criteria in the 2000 WHO classification (14) as 160 Glioblastomas (GB), 41 Anaplastic Astrocytomas (AA) and 20 Astrocytomas (AII). No mixed gliomas or tumours with an oligodendroglioma component were included. We have reviewed the Swedish Cancer Register data for the 2 regions from which the tumour material was collected and found that in both cases about 20% of patients who received a glioblastoma diagnosis during the period of collection were included in this study. The mean age was slightly lower than the mean for all cases in the cancer registry, as resection was necessary for inclusion. Patients not treated by gross total resection due to high age and/or complicating diseases could not be included. Data on the irradiation treatment details of 30 glioblastomas and 9 anaplastic astrocytomas patients was not retrievable at review. End of follow-up of the patients was set to October 1st 2003, providing a minimum follow-up time of patients alive at that date to 8.9 years. Clinical data were collected from the patient records at the hospitals where they were treated and/or followed up. The data included age and sex of the patient,

tumour localization, duration and type of symptoms before diagnosis, date of operation(s), post-operative radiotherapy and date of death. All operations were gross total, thus no cases diagnosed on biopsy alone were included. All patients gave informed consent and ethics committee approval for the project was obtained at all sites.

The paired tumour and blood samples were stored at -135°C or at -80°C for up to 5 years before DNA, and in the case of tumour tissue, RNA extraction. The majority of the tumours were included in previous studies and each tumour piece analyzed had, as assessed by histology, a minimum tumour cell content of 75%, but generally greater than 90% tumour cells. DNA and RNA from the tumour pieces and DNA from the patients' peripheral blood were extracted as described previously (21).

Gene copy number analysis: Southern Blotting and Quantitative PCR

Southern blotting—5 μg of 199 paired tumour and blood DNA was digested by the restriction enzyme *TaqI* and run into a 1% agarose gel and transferred to Hybond N+ membranes. Hybridization, using the pEP1 probe (genomic; approx 3kb; 1kb upstream and 2kb downstream of exon 1, which includes only intron 1 sequences) and a cDNA probe from the 3' non-coding region produced by RT-PCR amplification of bases 3901-4010 (GenBank, X00588) as well as PhosphorImager analysis were performed as described previously (16, 22). Probes that detect the highly informative polymorphic locus *D2S44* (pYNH24) were used as a control locus. Amplification was assessed by measuring the hybridization signals using Image Quant software and comparing the signals from the control locus and from *EGFR* as described (22).

Quantitative PCR to determine gene copy number—Real time quantitative PCR of tumour DNA using LightCycler (Roche) and SYBR Green I (Roche Molecular Biochemicals or Sigma) was performed on 22 tumours to determine the presence of amplification where there was limited DNA. PCR products of the wild-type *EGFR* gene were used in a dilution series to establish standard curves. In each reaction 1 ng of genomic DNA from each sample was used to determine *EGFR* copy number. Each assay was done in duplicate. The final analysis was performed using RelQuant (Roche) software. The *EGFR* region used for this quantitative PCR corresponds to 110 bases of exon 28 (which confers to bases 3901-4010 of the wild-type transcript) and is located at the 3' non-coding region. The control locus used here was WI-3360 and is located adjacent to the VNTR probe *D2S44* used in assessing amplification on the Southern blots (see above).

cDNA analysis

Generation of cDNA—The total RNA from each tumour sample was treated with 2 μl of RNase free DNase I (Invitrogen) at 37°C for 30 minutes followed by Phenol/Chloroform/Isoamyl-alcohol to remove DNase I. The DNA free RNA phase was precipitated and dissolved in RNasin/ H_2O and used to generate 100 μl of cDNA as follows. 3 μg of total RNA was mixed with 6 μl of random primer pdn6 (10ng/ μl) in a 20 μl volume, the mixture was denatured at 72°C for 10 minutes and cooled on ice immediately for 5 minutes. 5 μl of DTT (100mM), 5 μl of dNTP (10mM), 20 μl of 5 x first strand cDNA synthesis buffer were added, to make the volume up to 99 μl . This was then pre-warmed at 37°C for 2 minutes, 1 μl of Superscriptase III (Invitrogen; 200u/ μl) added, the solution mixed and incubated at 45°C for 2 hours. Then 1 μl of RNaseH was added to each cDNA sample and incubated at 37°C for 15 to 30 minutes.

Standard PCR—The PCR conditions for most experiments, except the discriminative PCR, have been described previously (21). In brief, a 10 minutes pre-denaturizing at 94°C , followed by a 35 cycles of 94°C for 30 sec - 55°C for 30 sec - 72°C for 1 minute, with a

final extension at 72°C for 10 minutes, using standard buffers and MgCl₂ concentration. The positions of the primers used and the sequence details are shown in Figure 1.

Discriminative PCR to differentiate wild-type transcript from *EGFRvIII* transcript

The forward primer used in the discriminative PCR was PC66, which is located in exon 1 and is the same as the forward primer used to examine 5' of *EGFR* transcript; the reverse primer PC1445 was designed so that the first two bases were complementary to the last two bases of exon 1 (274-275), followed by a further 20 bases complementary to the first 20 bases of exon 8 (1076-1095). The template consisted of 1 µl of cDNA solution. The PCR conditions were 10 minutes denaturizing at 94°C, followed by 35 cycles of; 94°C for 30 seconds, 61°C for 30 sec followed by 72°C for 1 minute, and finally extension at 72°C for 10 minutes.

Ribonuclease Protection Assay (RPA)—A cDNA fragment of bases 177–628 of the wild-type *EGFR* transcript was generated by PCR with PC66/PC1855. The reverse primer PC1855 has a T3 promoter sequence attached at its 5' end and the product was cloned into pBluescript II KS (–). A radioactive complementary RNA probe was produced using the T7 promoter and T7 reverse transcriptase, incorporating ³²P-dCTP, as recommended in the Ambion's Instruction Manual for MAXIscript™ In Vitro Transcription Kit. RPA was also performed according to these recommendations. In brief, 500 pg of ³²P labelled probe was mixed with 25 µg of total tumour RNA, NH₄OAC was added to a final concentration of 0.5 M, and the mixture of total tumour RNA and probe precipitated with ethanol. The pellets were re-suspended in 10 µl of hybridization solution from the kit, and 1 µl of 1% SDS added. The mixture was incubated at 94°C for 3 minutes, and then transferred to 42°C over night. 2 µl of RNaseA/RNaseT1 and 198 µl of RNase Digestion III buffer was mixed and 150 µl of this mixture added to each sample, incubated at 37°C for 30 minutes. The RNase digestion was terminated by adding 225 µl of Inactivation/Precipitation III solution. After precipitation, RNA pellets were dissolved in RNA sample loading buffer and run into 5% acrylamide/8M urea gel. The gels were dried and exposed to phosphor screens and scanned in a PhosphorImager and analyzed using ImageQuant software as described (16, 22).

Statistical analysis

All genetic data were categorized as either “normal” or “abnormal”. For *EGFR* gene amplification a minimum average *EGFR* gene copy number of 7 per genome was categorized as “abnormal”, if the average copy number was less the tumour was categorized as “normal”. If the aberrant transcript *EGFRvIII* was present, the tumour was categorized as “abnormal”. Absence of the *EGFRvIII* transcript was classified as “normal”. Other rearrangements or combinations of rearrangements were not considered. The genetic data was complete except for *EGFRvIII* data on 4 GBs due to too little amount of RNA available.

The starting point for patient follow-up was the date of the first operation, survival being reckoned from this date. All 160 GBs and 41 AA were included in the survival analyses. The AII patients were, due to the absence of *EGFR* aberrations and the fact that median survival was not reached, not included in the survival analysis.

For univariate analysis of single clinical or genetic factors and post-operative survival Wilcoxon-Gehan statistic (23) was performed. When a p-value <0.100 was seen in univariate analysis, Cox Regression analysis adjusting for age was performed. Stratification of GB patients (irradiated and non-irradiated, respectively) was done for sub-group analysis to explore if *EGFR* status could be associated with survival within a group of patients untreated after the operation or be a predictor of outcome after post-operative radiotherapy. When analyzing age and survival with age as a continuous variable Cox Regression analysis

was used. Comparing age distribution of patients with or without *EGFR* abnormalities was done with ANOVA test. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences), release 12.0.1.

Results

Clinical data

In Table 1A-B the clinical data of all GB and AA cases is summarized with median survival and some statistical comparisons. For the 160 GB patients median post-operative survival was 9.2 months and mean age 53.4 years. Only 2 GB patients were alive at end of follow-up. Age and post-operative irradiation were the only clinical factors showing significant survival differences. Age, analyzed both as a continuous variable and as a dichotomous variable, was inversely associated with survival ($p<0.001$). GB patients given post-operative irradiation survived significantly longer than those not irradiated ($p<0.001$). In bi-variate analysis using Cox regression both low patient age and radiotherapy were found to be independently associated with survival (both $p<0.001$; see Table 1a).

The median survival of the 41 AA patients was 4.6 years and the mean age 43.2 years. Twelve patients were alive at end of follow-up. As was found in the case of the GBs, patient age was inversely associated with survival. AA patients given radiotherapy post-operatively had a longer median survival than those not irradiated, but this difference was not statistically significant.

Amplification; incidence and degree

Tumours with an average *EGFR* gene copy number of 7 or more per genome were considered to have amplification of the gene. Amplification was found in 65 of 160 GBs (41%). None of the 20 AIIIs showed amplification, while 4 of 41 (9.8%) of the AAs did. The mean copy number in the amplified GBs was 44 copies (ranging from an average of 8 to 151 copies per genome).

Ten patients had been re-operated. One AII had progressed to an AA and one AA had progressed to a GB. There was no evidence of amplification in either case. Eight GBs were re-operated, 5 showed no evidence of amplification at either operation while 2 retained an amplified *EGFR* gene at the second operation. One GB had amplification only at the second operation. Another patient with a GB with *EGFR* amplification at the first operation, at re-operation had a tumour that was histologically classified as an AA but retaining the *EGFR* amplification. Representative cases of *EGFR* gene amplification status are shown in Figure 2A.

Identifying wild-type and *EGFRvIII* transcripts

EGFRvIII is the commonest aberrant *EGFR* form (found in up to 20% of GBs). While other aberrant forms exist, with either the extracellular or the cytoplasmic domain affected they have only been documented in single or very few cases. Using the primer pair PC66 and PC445 (see Figure 1), the presence of the wild-type *EGFR* transcripts was demonstrated in all 221 cases. When primer PC66 was paired with PC1403 (corresponding to bases 1093-1076) one band representing the wild-type transcript (923bp) could be identified in all samples, whereas the *EGFRvIII* transcript (122 bp) could be identified only in some tumours (Figure 2A-C).

In order to specifically identify even small amounts of the *EGFRvIII* transcript, a discriminative RT-PCR was designed using the PC66 primer paired with a primer (PC1445) with its two most 3' bases complementary to the last two bases in exon 1 (bases 275 and

274) and then the remaining 20 bases complementary to the first 20 bases in exon 8. This PCR, being specific for the *EGFRvIII* transcript, confirmed the previous findings and 34 GBs and 3 AAs with *EGFR* amplification showed a corresponding 125bp band (see GB13 in Figure 2C-D). In addition, 8 GB and 2 AA, that did not fulfil our criteria of *EGFR* amplification, showed the 125bp band by RT-PCR using the primers PC66 and PC1403, indicating that these tumours also produced the transcript for *EGFRvIII*. All AIs showed only a wild-type transcript product of 923bp (using the primer pair PC66 and PC1403). The results from representative cases are shown in Figure 2C-D.

An RNase Protection Assay was carried out in 6 cases where sufficient RNA was allowed, to confirm the PCR findings where the *EGFRvIII* transcript was identified, including tumours that did not fulfil the criteria for amplification of the gene (>5 copies). As described in Materials and Methods, a 605 base complement RNA molecule was produced to protect the wild-type and *EGFRvIII* transcripts resulting in the protection of an 458 base RNA sequence in the case of the wild-type transcript and a shorter 252 base sequence in the case of the *EGFRvIII* transcript (see Figure 3). The findings confirmed the PCR analysis in all the 6 cases studied. A GB xenograft known to highly express the *EGFRvIII* transcript was used as a positive control (Figure 3).

In total 42 GBs and 5 AAs expressed the *EGFRvIII* transcript. Of these 34/42 GBs (81%) and 3/5 AAs (60%) also fulfilled the criteria for *EGFR* amplification, while 29/63 (2 missing *EGFRvIII* data due to lack of RNA) GBs (46%) and 1/4 AAs (25%) with *EGFR* amplification showed no evidence of the *EGFRvIII* transcript.

EGFR gene abnormalities and survival; univariate and multivariate analyses

In the 160 GB patients we found no significant associations between *EGFR* abnormalities (*EGFR* amplification and expression of the *EGFRvIII* transcript, respectively) and survival (Table 2A-C). This was also tested in the sub-groups of irradiated and non-irradiated GB patients, with no significant association with survival found. We also performed univariate analysis setting up 3 groups of patients with: 1. Neither presence of *EGFRvIII* nor *EGFR* amplification; 2. Either presence of *EGFRvIII* or *EGFR* amplification; 3. Both presence of *EGFRvIII* and *EGFR* amplification. In univariate analysis none of these combinations provided any significant associations with survival (Table 3A-C).

In the 41 AA we found a tendency towards decreased survival times in the relatively few patients whose tumours had *EGFR* abnormalities. The 5 AAs expressing the *EGFRvIII* transcript, of which 3 also fulfilled the criteria of *EGFR* amplification, had a median survival of 1.3 compared to 5.6 years in the 36 cases not expressing *EGFRvIII* ($p=0,069$). None of the survival differences regarding the AAs was statistically significant in bivariate analysis adjusting for age (Table 2D and Table 3D).

EGFR gene abnormalities and age

In the 160 GB patients we found no significant associations between *EGFR* abnormalities and patient age (Table 4A-B). The AA patients with tumours with *EGFR* abnormalities had a higher mean age than those without. The age difference for AA cases with or without *EGFRvIII* was statistically significant ($p=0,023$) (Table 4C).

Discussion

The incidence of *EGFR* amplification in “primary” GBs (41%) and AAs (9.8%) is in accord with earlier reports (10, 15, 18, 19). The relative amounts of wild type and *EGFRvIII* transcripts varied from case to case, as reported previously (3, 18). In many tumours there was only wild type transcript whereas in others *EGFRvIII* or wild-type transcripts could

predominate. Many of the tumours required discriminative PCR for the demonstration of the *EGFRvIII* transcript indicating that the levels of its expression can be very low in some cases. This may well be related to the number of wild-type or mutated *EGFR* gene copies that are present (24). There has been some discussion in the literature whether the *EGFRvIII* transcript occurs in the absence of amplification. The numbers of cases of astrocytic gliomas reported without amplification but expressing the *EGFRvIII* have been small. To exclude the possibility that the finding is a PCR artefact and that the *EGFRvIII* transcript truly exists in the absence of amplification, the RNase protection assay was performed in 4 of the tumours without amplification and with an RT-PCR product indicative of the *EGFRvIII* transcript. The findings confirmed the presence of the *EGFRvIII* transcript. The *EGFR* gene has yet to be examined in detail in these cases to see if the *EGFR* copies present show gene rearrangements or other kinds of mutations or whether the *EGFRvIII* transcript is the result of aberrant splicing of a wild-type gene, even if the latter seems unlikely. However, it must also be remembered that in the cases where there was no convincing evidence of amplification and RT-PCR and RNase protection assays both showed evidence of the *EGFRvIII* transcript, amplification and rearrangement of amplified *EGFR* genes may have been present in a small subpopulation of cells in the tumour piece. The evidence of amplification from such sub-populations might be eclipsed by the main population of cells without amplification, yet the PCR technique might well pick up the aberrant transcript. There have been reports of many other aberrations of the *EGFR* transcript, particularly in GB, but these occur much more infrequently than the *EGFRvIII* variant.

EGFR gene abnormalities, particularly amplification, has been extensively studied and tested as a potential prognostic factor in high-grade astrocytic tumours. Reviewing the literature to-date reveals contradictory or inconclusive results. The meta-analysis carried out by Huncharek *et al* underscores this (20). The most recent and the to-date largest study on GB genetics and prognosis shows, as does our study, no association between survival and *EGFR* amplification (19). However, the presence or absence of transcript abnormalities was not reported.

In one recent study on 87 GB patients that did attempt to document both *EGFR* amplification and *EGFRvIII* (as detected by immunohistochemistry) the data showed a relatively small, but statistically significant, survival difference (1.2 compared to 1.7 years) in both univariate analysis and bivariate analysis adjusting for age in favour of patients without amplification. Further division of the amplified group revealed an association between *EGFRvIII* expression and even shorter median survival (18). In this study patients from different randomized treatment studies were included and some patients were only biopsied, which is a potential bias. In our study no combination of *EGFR* amplification and the presence of the *EGFRvIII* transcript in GBs was found to be associated with a good or bad prognosis. However our material was limited to craniotomy cases.

To explore if *EGFR* status could be associated with survival within a group of patients untreated after the operation or be a predictor of outcome after post-operative radiotherapy, sub-group analysis was performed but without significant findings. Thus, from this and previous studies it is reasonable to conclude that analysis of *EGFR* amplification and/or the presence of *EGFRvIII* are not a strong prognostic or predictive factor in conventionally treated GBs.

The difference in outcome between AA patients with or without *EGFR* amplification and *EGFRvIII*, respectively, was however more striking. All 5 patients with *EGFRvIII* had died before end of follow-up with a median survival of 1.3 years (compared to 5.6 years for AA patients with absence of *EGFRvIII*). Such a short survival is closer to what might be expected in a series of GBs, rather than a series of AAs. This indicates that there exists a

fraction of AA patients, whose tumours lack the histological characteristics of a GB, yet have a tumour form that shows a similar aggressive biological behaviour to a GB and that these cases may be identified by this kind of genetic analysis. The fact that the survival difference was not statistically significant in multivariate analysis probably reflects the small numbers of AAs studied. In a recent study of EGFRvIII with immunohistochemical techniques EGFRvIII positivity was found to be associated with poor outcome in AA patients in univariate analysis, while no such association was found in GB patients (25). They also found EGFRvIII positivity associated with high age in the AA group, an association not found in the GB group. The study by Shinojima, *et al*, also presented data indicating the association of particular *EGFR* abnormalities and patient age (18). As presented in Table 4A-C, in our series of GB patients we found no such associations, but the AA patients with *EGFR* abnormalities were older than those without.

During the last 2 decades, the awareness of the high frequency of genetic alterations and/or over-expression of *EGFR* in GB and other tumour forms has lead to the development of different treatment strategies targeting EGFR. These include passive immunotherapy using monoclonal antibodies against wild-type EGFR and/or the EGFRvIII variant (26-29), radioimmunotherapy (30), small molecule tyrosine kinase inhibitors (31, 32) and radiolabeled or toxin-conjugated EGFR ligands (33, 34). In order to choose the optimal therapy from these new developments for the individual patient, one will have to consider the affinity and specificity of the molecular therapy for the patient's tumour cells. Thus, the use of EGFR targeting therapies in clinical practice, will require an analysis of the *EGFR* copy number, *EGFR* expression level, possible rearrangements and point mutations, resulting in aberrant transcripts and proteins. While the present findings indicate that a molecular analysis of the *EGFR* gene is of little prognostic value with conventional therapy, at least in the case of AII of GB, it may become essential for adequate treatment decisions regarding novel molecular targeted therapies in the near future.

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Figure 1.

A diagram showing the 5' end of the wild-type *EGFR* transcript, coding for the extracellular domain, with all probes and primers used in the study. RPA=Ribonuclease Protection Assay; TM=sequence coding for the transmembrane domain. The arrowhead indicates the 3' end of each primer.

Figure 2.

A-D. A) The radiogram of blood (=B) and tumour DNA (=T) pairs on *TaqI* Southern blots probed with the pEP1 probe (encompasses 1000 bp upstream of *EGFR* exon 1, exon 1 itself and approximately 2000 bp of intron 1). Note the large signal difference between B and T DNA in cases GB23, GB45, GB47, GB242 and GB248 indicating *EGFR* amplification. Control probing of the same blots with other chromosome 7 and chromosome 2 probes showed loading of DNA to be approximately equal for each of the B and T pairs. B-C) PCR products using PC66 and PC1403 from some of the AA and GB cases. The case number is given above each lane together with *EGFR* amplification status (“+” for amplified; “-” for non-amplified). Note that while all tumours show a 923 bp product (indicating presence of the wild-type transcript) some also show a 122 bp product (indicative of the *EGFRvIII* transcript). A product of the *EGFRvIII* transcript is shown for GB6 (which had *EGFR* amplification) as well as in AA94, GB128 and GB13 (which did not fulfil our criteria for *EGFR* amplification). wt=wild-type; bp=base pair. D) Discriminative RT-PCR using primers PC66 and PC1445. The RNA used was from the same cases shown in Figure 2C and demonstrates the specificity of the reaction with only GB13 showing a positive result (a 125 bp band) from this set of tumours.

Figure 3.

Ribonuclease Protection Assay (RPA) demonstrating the presence of both the wild-type and the *EGFRvIII* transcript. A813 is a 4th passage of xenograft with an amplified and rearranged *EGFR* gene, which mainly express the *EGFRvIII* transcript. GB182 is a GB without amplification of the *EGFR* gene but that express the *EGFRvIII* transcript, as is demonstrated here. GB180 and AA108 are tumours with *EGFR* amplification that only express the wild-type transcript.

Table 1
Clinical data, median post-operative survival and univariate analysis

A. The 160 GB.				
	No. of cases	Median survival (years)	p-value ^a	p-value ^b
Overall post-operative survival	160	0.8		p<0,001 ^c
Age in relation to survival (Mean age: 53.4 years)				
Age groups				
<60 years	80	0.9		p<0,001
60 years	80	0.5		
Sex				
Female	59	0.7		p=0,633
Male	101	0.8		
Localization				
Cerebral	158	0.8		p=0,818
Infratentorial	2	0.8		
Side				
Left	80	0.8		p=0,167
Right	80	0.6		
Post-operative radiotherapy				
Yes	80	1.0		p<0,001
No	50	0.4		
p<0,001				
B. The 41 AA.				
	No. of cases	Median survival (years)	p-value	
Overall post-operative survival	41	4.6		p=0,009 ^c
Age in relation to survival (Mean age: 43.2 years)				
Age groups				
<40 years	19	5.8		p=0,007
40 years	22	1.7		
Sex				
Female	26	4.1		p=0,612
Male	15	6.0		
Localization				
Cerebral	38	4.5		p=0,389
Infratentorial	3	9.2		
Side ^d				
Left	17	4.6		p=0,655
Right	20	4.5		

B. The 41 AA.

		No. of cases	Median survival (years)	p-value
Post-operative radiotherapy	Yes	21	4.6	p=0.255
	No	11	3.0	

^aUnivariate analysis of post-operative survival using Wilcoxon-Gehan.

^bBivariate analysis adjusting for age using Cox Regression.

^cCox Regression analysis with age as a continuous variable.

^dFour cases with central location of the tumor excluded from this analysis.

Table 2
Median post-operative survival in years in cases with or without the specified *EGFR* abnormalities^a

A. All the 160 GB.		
	<i>EGFRvIII</i>	<i>EGFR</i> amplification
Median survival in cases without the specified <i>EGFR</i> abnormality	0,6	0,6
(n=)	(114)	(95)
Median survival in cases with the specified <i>EGFR</i> abnormality	0,9	0,9
(n=)	(42)	(65)
Wilcoxon-Gehan p=	0.357	0.083
Cox Regression^b p=	-	0.278
B. The 80 GB treated with post-operative radiotherapy.		
	<i>EGFRvIII</i>	<i>EGFR</i> amplification
Median survival in cases without the specified <i>EGFR</i> abnormality	0,9	0,8
(n=)	(54)	(46)
Median survival in cases with the specified <i>EGFR</i> abnormality	1,2	1,1
(n=)	(23)	(34)
Wilcoxon-Gehan p=	0.508	0.210
C. The 50 GB not treated with radiotherapy.		
	<i>EGFRvIII</i>	<i>EGFR</i> amplification
Median survival in cases without the specified <i>EGFR</i> abnormality	0,5	0,5
(n=)	(38)	(33)
Median survival in cases with the specified <i>EGFR</i> abnormality	0,3	0,3
(n=)	(11)	(17)
Wilcoxon-Gehan p=	0.422	0.556
D. The 41 AA.		
	<i>EGFRvIII</i>	<i>EGFR</i> amplification
Median survival in cases without the specified <i>EGFR</i> abnormality	5,4	5,3
(n=)	(36)	(37)
Median survival in cases with the specified <i>EGFR</i> abnormality	1,3	1,4
(n=)	(5)	(4)
Wilcoxon-Gehan p=	0.069	0.317
Cox Regression^b p=	0.343	-

^aFor definitions of the *EGFR* abnormalities, see Materials and Methods.

^bCox Regression multivariate analysis adjusting for age

Table 3
Median post-operative survival in years in cases with or without combined *EGFR* abnormalities^a

A. All the 160 GB.	
Median survival in cases with neither <i>EGFRvIII</i> nor <i>EGFR</i> amplification (n=85)	0.6
Median survival in cases with either <i>EGFRvIII</i> or <i>EGFR</i> amplification (n=37)	0.8
Median survival in cases with both <i>EGFRvIII</i> and <i>EGFR</i> amplification (n=34)	0.9
Wilcoxon-Gehan p=	0.259
B. The 80 GB treated with post-operative radiotherapy.	
Median survival in cases with neither <i>EGFRvIII</i> nor <i>EGFR</i> amplification (n=39)	0.8
Median survival in cases with either <i>EGFRvIII</i> or <i>EGFR</i> amplification (n=20)	1.1
Median survival in cases with both <i>EGFRvIII</i> and <i>EGFR</i> amplification (n=18)	1.2
Wilcoxon-Gehan p=	0.506
C. The 50 GB not treated with radiotherapy.	
Median survival in cases with neither <i>EGFRvIII</i> nor <i>EGFR</i> amplification (n=31)	0.5
Median survival in cases with either <i>EGFRvIII</i> or <i>EGFR</i> amplification (n=9)	0.4
Median survival in cases with both <i>EGFRvIII</i> and <i>EGFR</i> amplification (n=9)	0.4
Wilcoxon-Gehan p=	0.721
D. All 41 “primary” AA.	
Median survival in cases with neither <i>EGFRvIII</i> nor <i>EGFR</i> amplification (n=35)	5.3
Median survival in cases with either <i>EGFRvIII</i> or <i>EGFR</i> amplification (n=3)	7.3
Median survival in cases with both <i>EGFRvIII</i> and <i>EGFR</i> amplification (n=3)	1.3
Wilcoxon-Gehan p=	0.169

^aFor definitions of *EGFR* abnormalities, see Materials and Methods.

Table 4
Mean age in cases with or without the specified *EGFR* abnormality^a. ANOVA test

A. The 160 GB.		
	<i>EGFRvIII</i>	<i>EGFR</i> amplification
Mean age in cases without the specified <i>EGFR</i> abnormality	56,6	55,9
(n=)	(114)	(95)
Mean age in cases with the specified <i>EGFR</i> abnormality	57,5	57,6
(n=)	(42)	(65)
ANOVA p=	0.686	0.394
B. The 160 GB.		
Mean age in cases with neither <i>EGFRvIII</i> nor <i>EGFR</i> amplification (n=85)	56.5	
Mean age in cases with either <i>EGFRvIII</i> or <i>EGFR</i> amplification (n=37)	56.5	
Mean age in cases with both <i>EGFRvIII</i> and <i>EGFR</i> amplification (n=34)	57.9	
ANOVA p=	0.834	
C. The 41 AA.		
	<i>EGFRvIII</i>	<i>EGFR</i> amplification
Mean age in cases without the specified <i>EGFR</i> abnormality	41,0	42,5
(n=)	(36)	(37)
Mean age in cases with the specified <i>EGFR</i> abnormality	59,0	49,5
(n=)	(5)	(4)
ANOVA p=	0.023	0.440

^aFor definitions of *EGFR* abnormalities, see Materials and Methods.