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Expression of the epidermal growth factor receptor in astrocytic tumours is specifically associated with glioblastoma multiforme

Reto M. Agosti[1], Margrit Leuthold[2], William J. Gullick[4], M. Gazi Yasargil[3], and Otmar D. Wiestler[1,*]

[1] Institute of Neuropathology, Department of Pathology, [2] Department of Internal Medicine and [3] Department of Neurosurgery, University Hospital, Zürich, Switzerland
[4] Protein Chemistry Laboratory, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, UK

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Summary. Epidermal growth factor and its receptor (EGFR) constitute an important and well-characterized mitogenic system in various ectodermal tissues including glial cells. Over-expression of the EGFR due to gene amplification has been reported in primary brain tumours of glial origin. Using a monoclonal antibody to the EGFR and immunohistochemical analysis, we examined the expression and distribution of EGFR in 103 astrocytic tumours. In addition, selected tumours were studied by Western blotting using a polyclonal antibody to EGFR and by Southern blot analysis. Glioblastomas (WHO grade IV) showed EGFR expression in 37% of cases, whereas pilocytic (WHO grade I), low-grade (WHO grade II) or anaplastic astrocytoma (WHO grade III) were invariably EGFR negative. Generally, there was a close correlation between the presence of EGFR gene amplification and over-expression of receptor protein. Different patterns of immunoreactive cells and significant intratumour heterogeneity of EGFR expression were observed in glioblastomas. The specific association of EGFR over-expression with glioblastoma may provide a useful diagnostic tool for distinguishing anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (WHO grade IV).

Key words: Astrocytoma – Glioblastoma multiforme – Oncogenes – Epidermal growth factor receptor – Immunohistochemistry

Introduction

The epidermal growth factor receptor (EGFR) represents a 170 kDa transmembrane glycoprotein which has been identified on a variety of ectodermal cell types, including glial cells. The receptor molecule is composed of a large *N*-terminal extracellular domain which harbours the ligand binding site, a transmembrane anchor and a cytoplasmic protein with an intrinsic protein-tyrosine kinase activity (Hunter and Cooper 1985). Binding of epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and other ligands to the EGFR activates the protein-tyrosine kinase, and it is believed that this receptor activation results in a specific cellular response to EGF. The human EGFR has been purified to homogeneity from normal human placenta and from the human vulval carcinoma cell line A431 (Downward et al. 1984).

The EGFR gene is the cellular counterpart of the retroviral v-*erb*B_1 oncogene of avian erythroblastosis virus. The viral *erb*B_1 protein lacks most of the extracellular domain which renders the protein-tyrosine kinase constitutively active and ligand independent. Genomic amplification of the EGFR gene with over-expression was first observed in the A431 squamous carcinoma cell line (Libermann et al. 1984, 1985). More recently, a high incidence of EGFR amplification has been reported in human malignant gliomas and glioma cell lines (Arita et al. 1989; Bigner and Vogelstein 1990; Bigner et al. 1987; Bruce et al. 1988; Ekstrand et al. 1991; Gerosa et al. 1989; Hall et al. 1990; Hawkins et al. 1991; Hunter and Cooper 1985; Malden et al. 1988; Reifenberger et al. 1989; Reubi et al. 1989; Ro et al. 1988; Strommer et al. 1990; Torp et al. 1991). In the present study, we have analysed a large series of astrocytic neoplasms in order to establish the incidence of EGFR expression in these tumours and to examine a potential correlation of the EGFR status with the grade of malignancy.

Materials and methods

We collected tissue samples from a consecutive series of 103 human astrocytic brain tumours. This series was composed of 21 pilocytic astrocytomas (WHO grade I), 10 well-differentiated astrocytomas (WHO grade II), 26 anaplastic astrocytomas (WHO grade III) and 46 cases of glioblastoma multiforme (WHO grade IV). Histopathological diagnoses were based on the WHO brain tumour classification (Kleihues et al. 1992; Zülch 1979).

* *Present address:* Institute of Neuropathology, University of Bonn, Sigmund-Freud-Str. 25, W-5300 Bonn 1, Federal Republic of Germany

Offprint requests to: O.D. Wiestler, at his present address

The tumour samples were processed immediately after surgery. One piece was frozen in liquid nitrogen and stored at −70° C for molecular analysis. The remainder of the tissue was fixed overnight in 4% buffered formaldehyde solution, embedded in paraffin, sectioned and stained with haematoxylin and eosin for diagnostic purposes. For immunohistochemical studies, all tumours were examined with antibodies to glial fibrillary acidic protein (GFAP; polyclonal rabbit antibody; Dakopatts, Copenhagen, Denmark) and to the EGFR. A monoclonal mouse antibody (F4) raised against a synthetic intracellular 12aa peptide corresponding to residues 985–996 of the human EGFR was used to detect EGFR (Gullick et al. 1986; Kris et al. 1985). This antibody has previously been applied successfully on paraffin sections (Gullick et al. 1986). The sections were incubated with a 1:40 dilution of the F4-antibody in phosphate buffered saline (PBS) (0.05 M, pH 7.4–7.6) at room temperature for 1 h. To localize the primary antibody, a three-step peroxidase-antiperoxidase procedure was used (Sternberger 1982). In order to establish the reaction conditions the antibody was tested on sections of a nude mouse tumour, produced by subcutaneous injection of A-431 cells, an established vulval carcinoma cell line with documented amplification and over-expression of the EGFR gene. The specificity of the antibody was confirmed by pre-incubation of F4 with the synthetic peptide antigen. This abolished the immunoreaction.

DNA was extracted from frozen tumour tissues by phenol extraction and ethanol precipitation (Ausubel et al. 1987). Purified genomic DNA was digested with the restriction enzyme *Hin*dIII, separated on a 1% agarose gel and transferred to a Gene Screen Plus membrane (NEN Research Products, Regensdorf, Switzerland). A cDNA probe complementary to an internal fragment of the EGFR gene (generously provided by J. Filmus and R. Buck, Toronto) was labelled with ^{32}P according to the method of Feinberg and Vogelstein (1983); hybridization and washing were performed following established protocols (Maniatis et al. 1989). A probe complementary to the human β-actin gene was used to demonstrate comparable amounts of DNA on all lanes (data not shown). The extent of EGFR gene amplification was determined by densitometry scanning of the autoradiographs (see legend to Fig. 1).

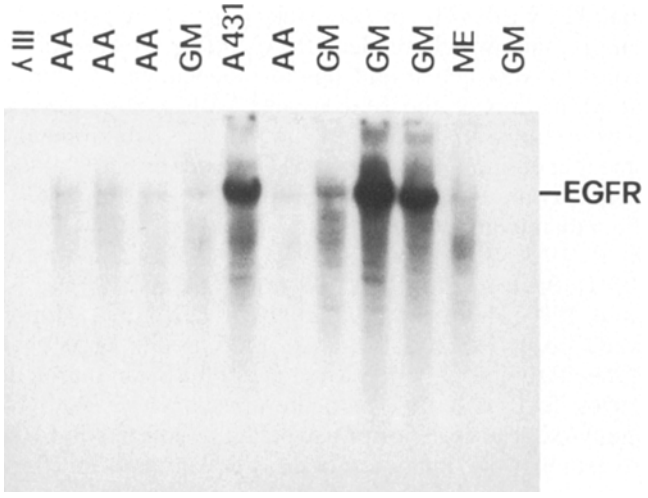

Fig. 1. Southern analysis of the epidermal growth factor receptor (*EGFR*) gene. Genomic tumour DNAs (15 µg) were digested with *Hin*dIII. The blots were hybridized with a cDNA probe complementary to an internal fragment of the EGFR gene. Lanes: Unlabelled molecular size standards (*λIII*), A431 squamous carcinoma cells (*A431*), glioblastoma multiforme (*GM*), anaplastic astrocytoma (*AA*), meningioma (*ME*). The A431 carcinoma cell line (*lane 6*) and two glioblastomas (*lanes 9 and 10*) show a 21-, 20- and 52-fold amplification of the EGFR gene, respectively. Protein extracts of the tumours in lanes 3 and 5 were also analysed in the Western blot experiment shown in Fig. 3 (*lanes 3 and 6*, respectively)

For protein extraction, frozen tumour tissue was homogenized in 10 volumes of extraction buffer [50 mM TRIS/HCl, pH 6.8, 1% Triton X-100, 5 mM ethylene glycol bis-(β-amino ethyl ether) tetraacetic acid (EGTA), 3 mM phenylmethylsulphonyl fluoride, 50 µM leupeptin]. Following centrifugation at 12,000 *g* (4° C, 10 min) supernatants were denatured (100° C, 5 min) in 2 × electrophoresis sample buffer (100 mM TRIS/HCl, pH 6.8, 4% sodium dodecyl sulphate, 10% glycerol, 40% β-mercaptoethanol, 20 mM ditiothreitol) and stored at −70° C. The pellets were denatured in 1 × electrophoresis sample buffer.

For electrophoretic separation of cellular proteins, a 7.5% polyacrylamide gel was used. Approximately 15 µg of total protein was applied to each well. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and protein transfer onto nitrocellulose paper (Ba85; Schleicher and Schuell, Feldbach, Switzerland) were carried out in a Bio-Rad Mini Protean electrophoresis and blotting system. Following pre-incubation in PBS containing 5% non-fat milk powder (Sanolait, COOP, Switzerland), the membranes were incubated in the same solution containing a polyclonal rabbit antibody to EGFR at a 1:40 dilution. The antibody was visualized with alkaline phosphatase-conjugated, affinity-purified immunoglobulins to rabbit IgG (1:500) and the Bio-Rad alkaline phosphatase substrate kit.

Results

The expression of EGFR was studied immunohistochemically in paraffin sections of 103 primary glial brain tumours. Our series included 21 pilocytic astrocytomas (WHO grade I), 10 well-differentiated astrocytomas (WHO grade II), 26 anaplastic astrocytomas (WHO grade III) and 46 glioblastomas (WHO grade IV). Expression of the EGFR was found in 37% of the glioblastoma samples. However, none of the astrocytomas, of WHO grades I, II and III, showed immunoreactivity for EGFR protein (Table 1).

Glioblastomas with EGFR expression showed a striking regional heterogeneity in the distribution of receptor protein (Fig. 2). In many instances, the reactions were confined to circumscribed areas of the tumour which were not distinguishable from the remaining tissue by morphological criteria. This may indicate clonal expansion of cells with EGFR amplification. Other tumours exhibited a more scattered pattern of EGFR-positive cells. The predominant cell type immunoreactive for the receptor were small, poorly differentiated tumour cells. Occasionally, a positive reaction was also seen in giant cells. None of the glioblastomas included in this series showed uniform expression of EGFR. Representative patterns of immunoreactivity are shown in Fig. 2.

Table 1. Epidermal growth factor receptor (EGFR) expression in astrocytic tumours

Tumour	EGFR expression[a]
Pilocytic astrocytoma (WHO grade I)	0% (0/21)
Astrocytoma (WHO grade II)	0% (0/10)
Anaplastic astrocytoma (WHO grade III)	0% (0/26)
Glioblastoma multiforme (WHO grade IV)	37% (17/46)

[a] Paraffin sections were studied immunohistochemically using the F4 antibody to EGFR

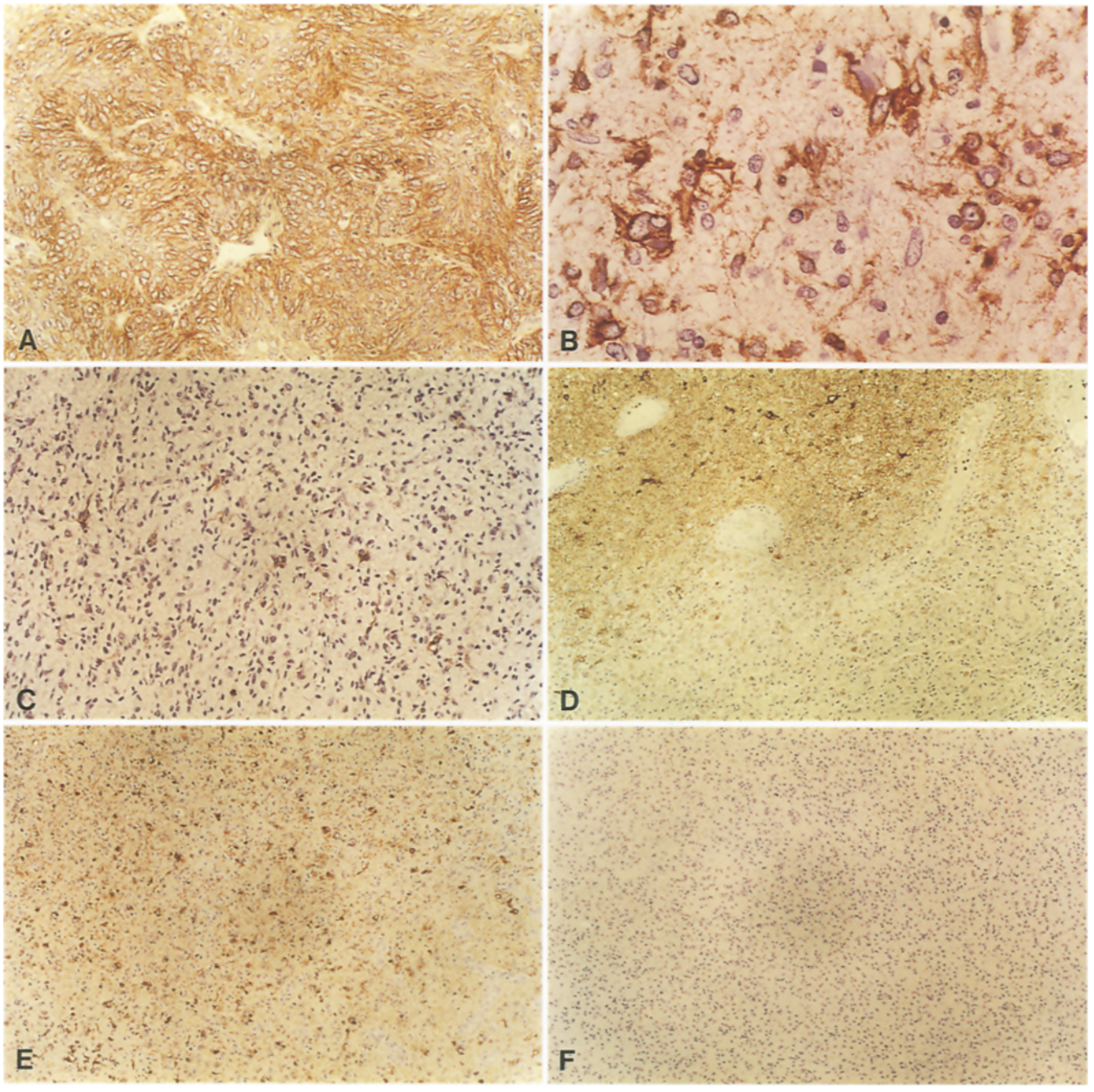

Fig. 2 A–F. Immunohistochemical analysis of EGFR protein on paraffin sections. **A** A431 nude mouse tumour with an amplified EGFR gene (positive control). **B–E** Representative glioblastoma samples. Note the variable patterns of EGFR immunoreactivity. **F** Same tumour as **E**. The reaction was abolished by pre-incubation of the F4 antibody with the corresponding synthetic peptide (specificity control). **A** ×250; **B** ×400; **C** ×150; **D–F** ×100

In a selected series of samples, Western blot analysis was carried out with a polyclonal antibody raised against the identical polypeptide used to generate the F4 monoclonal antibody. These tumours were also analysed by Southern blotting for EGFR gene amplification. The association between amplification of the EGFR gene and the expression of EGFR protein was first established in a nude mouse tumour induced by s.c. injection of A431 cells (Fig. 2). In all glioblastoma specimens examined, there was a good correlation between EGFR gene amplification, the amount of EGFR protein detectable by immunoblotting and immunohistochemical recognition of the receptor on paraffin sections. Only 1 glioblastoma showed amplification of the EGFR gene without significant immunoreactivity for receptor protein. Representative Western and Southern blots are shown in Figs. 1 and 3. We did not find evidence for rearrangement of the EGFR gene or abnormal EGFR polypeptides.

In an attempt to correlate the activated EGFR pathway with morphological features of the respective tumours, the predominant tumour cell type, vascular endo-

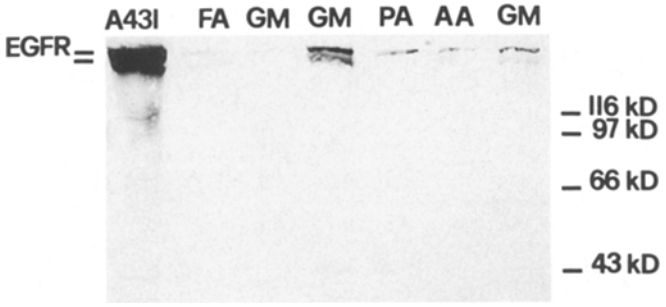

Fig. 3. Western blot analysis of the EGFR protein in tumour extracts. A polyclonal antipeptide antibody to an internal domain of the EGFR protein was used. Each lane contains 15 µg of protein extracted from A431 squamous carcinoma cells (*A431*), glioblastoma multiforme (*GM*), anaplastic astrocytoma (*AA*), fibrillary astrocytoma (*FA*) and pilocytic astrocytoma (*PA*). Note high levels of EGFR protein in A431 cells and in one glioblastoma (*lane 4*)

thelial proliferation, mitotic activity, necrotic change and immunoreactivity for GFAP were analysed in all glioblastomas included in this study. None of these variables was found to be associated with the pattern of EGFR expression.

Discussion

This study was initiated to determine the incidence and the patterns of EGFR expression in a large series of low-grade astrocytomas, anaplastic astrocytomas and glioblastomas. Following the original reports by Libermann et al. (1984, 1985) several laboratories have described amplification of the EGFR gene and/or overexpression of EGFR protein in malignant gliomas. The studies by Strommer et al. (1990) and Ekstrand et al. (1991) found evidence for EGFR gene amplification in approximately 50% of glioblastomas, the majority of which also showed high levels of EGFR mRNA. In our study and in the report by Ekstrand et al. (1991), there was a close correlation between the presence of an amplified EGFR gene, elevated levels of EGFR mRNA and the immunohistochemical detection of the gene product in tissue sections. However, other investigators have reported significantly higher incidences of EGFR-positive gliomas using immunohistochemical techniques (Reifenberger et al. 1989) or Southern blot analysis (Bigner et al. 1987; Strommer et al. 1990). This discrepancy is not entirely resolved. The use of different antibodies to EGFR, differences in tissue processing and sampling errors may account to some extent for divergent results. The monoclonal antibody F4 employed in the present study is one of the few reagents capable of detecting EGFR on paraffin sections. In normal brain and *low*-grade gliomas, F4 does not usually yield positive staining, and there is no evidence for cross-reactivity with other cerebral proteins. As this antibody was raised against a synthetic peptide, the specificity of the immunohistochemical staining can be controlled by peptide inhibition. High incidences of EGFR binding in malignant gliomas have also been reported in several studies which employed receptor autoradiography with radiolabelled EGF (Hawkins et al. 1991; Reubi et al. 1989). A potential pitfall with this technique is the detection of alternative receptors for EGF.

All glioblastoma samples with immunoreactivity for EGFR displayed a striking intratumour heterogeneity of expression. The predominant pattern of protein distribution was characterized by a combination of areas with a high number of positive cells and of virtually or completely negative tumour components. This expression pattern would be compatible with a clonal expansion model of EGFR-positive neoplastic cells during the progression stage of glioma development. However, we have also observed occasional glioblastomas with a more scattered distribution of immunoreactive cells (Fig. 2). Such a pattern may imply regulation of the EGFR gene by an as yet unknown mechanism within the tumour. We looked for evidence that overproduction of EGFR might affect the histopathological appearance of individual glioblastomas. However, attempts to correlate immunoreactivity for EGFR with specific histopathological features of the tumours such as predominance of a specific cell type, immunohistochemical expression of GFAP, mitotic activity, vascularization and the extent of necrosis were all unsuccessful.

A significant finding of the present study on astrocytic tumours is the exclusive association of EGFR expression with glioblastoma multiforme (WHO grade IV). This may indicate that alterations of the EGFR pathway are usually acquired during an advanced stage in the evolution of malignant gliomas. Deregulation of the EGFR signal could also represent a determinant of the aggressive biological behaviour of glioblastomas. We have recently demonstrated that another genetic defect characteristic of glioblastoma multiforme; a loss of genetic material on chromosome 10 is frequently associated with amplification of EGFR (von Deimling et al., in press). To what extent these changes co-operate and which alternative transforming mechanisms substitute for EGFR in glioblastomas without an amplified and over-expressed EGF receptor remains to be determined. The failure to detect EGFR protein in tissue specimens of pilocytic astrocytomas (WHO grade I), low-grade astrocytomas (WHO grade II) and anaplastic astrocytomas (WHO grade III) has interesting diagnostic implications as a positive immunohistochemical reaction appears to provide strong evidence for a diagnosis of glioblastoma multiforme. In traditional surgical neuropathology, the presence of necrotic areas within an anaplastic astroglial neoplasm is the strongest indicator of glioblastoma. However, this feature may be missing in small tumour specimens such as stereotaxic biopsies. In order to further substantiate the association of EGFR and glioblastoma multiforme, it will be necessary to study an even larger series of tumours.

In conclusion, we have observed a remarkable specific association of the EGFR oncogene with a subset of glioblastoma multiforme. This finding points to a specific role of the EGFR pathway in the evolution of these tumours and implicates this receptor protein as a molecular marker for glioblastomas.

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