both mutant and wild type alleles. An algorithm based on the ASRA technique to determine the proportion of mutant to wild type sequences is currently being evaluated (Iland, H., unpublished results).

In conclusion, the low frequency of *ras* mutations expressed in melanoma suggests that they do not play a major role in this disease but may reflect genetic instability of the *ras* gene or of the genome in general. Our results do support the correlation between exposure to sunlight and N-*ras* codon 61 mutations in cutaneous melanoma.


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# EGF Receptor Amplification and Expression in Human Brain Tumours

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**Human epidermal growth factor receptor (EGFr) gene amplification, rearrangements and expression were studied in tumours of the human nervous system. EGFr gene amplification was studied in 46 brain tumours. Gene expression was analysed by northern blot in 37 tumours and binding of its protein to EGF in 27 tumours. The *EGFr* gene was simultaneously amplified (with arrangements in 12.5% of gliomas) and overexpressed in 53% (9/17) of malignant gliomas, but never in meningiomas. In five high grade gliomas, amplification was always associated with a high level of receptors. However, since high amounts of EGF receptors found in one glioma were not the result of gene amplification, several systems of deregulation in EGFr production may exist and could be located at translational and/or post-translational levels.**
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## INTRODUCTION

THE HUMAN epidermal growth factor receptor (EGFr) is a single-chain transmembrane glycoprotein of molecular weight 170 kDa. The receptor includes an extracellular ligand binding domain, a transmembrane sequence, and an intracellular portion which has tyrosine kinase activity [1]. EGFr is activated by binding to EGF or EGF-like factors such as transforming growth factor alpha (TGFα). The v-*erb*B oncogene [2] encodes a truncated form of EGFr, and the loss of the extracellular portion results in constitutive phosphorylation of the receptor [3].

The EGFr gene is amplified and overexpressed in various tumoural tissues including brain neoplasia and human epidermoid carcinoma cells [4–6]. High EGF receptor levels have been observed in human gliomas [7, 8], and EGFr gene amplification has been described in 40% of glioblastomas [9, 10]. Abnormalities of EGFr have been found in the human glioblastoma cell line SF268 [11] in which an amplified EGFr gene appears to encode an enzymatically inactive protein [12]. EGF and TGF-α are the main peptides which bind EGFr. It has been shown that they have proliferating potential [13, 14].

The purpose of this work was to study a possible implication of the EGFr gene in human nervous system tumours. Amplification, mutations, and expression of EGFr gene as well as

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binding of EGF receptors to EGF were investigated in various human brain tumours. By means of this study, from gene to protein, we suggest that among 27 tumours, there may exist more than one pathway leading to an overproduction of EGF receptors.

## MATERIALS AND METHODS

*Human brain tumours*

54 tumours (Table 1) were obtained during neurosurgical procedures, immediately frozen in liquid nitrogen, and stored in sterile conditions at −70°C until use. Among the 54 specimens, 28 gliomas of different histological types and grades were analysed simultaneously for longitudinal study from gene to protein (Table 2). Normal brain tissues (non infiltrated peritumoural tissue) and human leucocytes were used as controls.

*Cell cultures*

Human vulvae carcinoma (A431) and murine adrenocortical tumour (Y1) cell lines were used as positive and negative controls respectively. Cells were grown, in monolayer, in Dulbecco's modified Eagle medium (DMEM) [supplemented with 10% fetal bovine serum, penicillin, streptomycin, Ham medium (F12) (v/v), and gentamicin; the final concentration of glutamine was 4 mmol/l] at 37°C and 5% $CO_2$. Cells were harvested during the exponential growth phase.

*DNA isolation*

High molecular weight DNAs were prepared by the usual techniques of phenol purification and ethanol precipitation as described in our previous studies [15].

*RNA isolation*

Total RNA was prepared from the same tumours using Chirgwin *et al*'s. method [16].

*Probes*

The cDNA EGFr probe used was the 1.6 kilobase *Eco*RI fragment of pE7 [17] labelled by random priming with α-[$^{32}$P] dCTP (Amersham).

*Southern blot analysis*

The DNA isolated from 46 tumours (25 gliomas, 13 meningiomas, 6 cerebral metastases, 1 medulloblastoma, and 1 neuroblastoma) was digested with restriction endonuclease *Eco*RI, separated by electrophoresis in 0.7% agarose gel, and transferred to a nylon membrane (Amersham) using Southern's method [18]. DNAs were fixed by a 2 min UV illumination. Hybridisation was performed at 42°C for 24 h in 20 mmol/l phosphate pH 6.8, 1× Denhardt's solution [0.02% bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone, 0.02% Ficoll], 0.1% SDS (sodium dodecyl sulphate), 5× SSC, (saline sodium citrate), 200 μg/ml denatured herring sperm DNA, and 50% formamide. The membrane was initially washed 4 times for 5 min in 2× SSC/0.1% SDS at room temperature then 1–3 times for 20 min in 0.1× SSC/0.1% SDS at 42°C. The hybridised filters were exposed to Agfa XRP1 film for 1–6 days at −70°C using intensifying screens. Hybridisation with a β-globin probe (1.0 kilobase *Eco*RI restriction fragment from plasmid pBR322) was used as an internal control for gene amplification. The intensity of the amplified bands was calculated as a gene to β-globin ratio and compared with normal (peritumoral) brain. The A431 cells in which EGFr gene amplification was known to be 30-fold [5] (confirmed by dot blot assays) were also used to quantify the level of amplification in the tumours by serial dilutions and densitometric analysis.

*Northern blot analysis*

To isolate total RNAs, 37 tumours (19 gliomas, 12 meningiomas, 5 cerebral metastases, and 1 neuroblastoma) were denatured in 50% formamide and 6.2% formaldehyde, for 15 min at 65°C, run on 1% agarose–formaldehyde gel, and transferred to nylon sheets (Amersham). RNAs were fixed by a 2 min UV illumination. Filters were prehybridised for 4 h at 42°C in 50% formamide, 5×SSPE, 5×Denhart's solution, 0.5% SDS, and 200 μg/ml denatured herring sperm DNA. The filters were then incubated 24 h in fresh buffer with 12.5 ng/ml of cDNA EGFr probe (specific activity: 6–7 × $10^8$ cpm/μg). After hydridisation, filters were then washed at high stringency (to 0.1×SSPE/0.1% SDS for 1 h at 42°C). The hydridised filters were exposed to Agfa XRP1 film for 1–6 days at −70°C using intensifying screens. The hybridisation signal with a human β-actin probe (1.2 kilobase *Pst*I restriction fragment from plasmid pBR322) was used as control for the amount of mRNA. The intensity of mRNA bands in various types of tumours was calculated as a β-actin to gene ratio which was compared with peritumoural brain tissue and mRNA of the A431 cell line. For sequential RNA–DNA hydridisation of the same filter using different probes, hybrids were removed from the filters by incubation at 65°C in 5 mmol/l Tris–HCl pH 8.0, 2 mmol/l EDTA, 0.1 × Denhart's solution.

*Partial purification of membrane extracts*

Frozen tissue samples (about 1g) were homogenised in 4 vol (w/v) of buffer containing 20 mmol/l *N*-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonate) (Hepes) at pH 7.4, 1.5 mM $MgCl_2$, 1 mmol/l ethylene glycolbis (β-aminoethyl ether)*N*, *N*, *N'*, *N'*-tetra-acetate (EGTA), 10 μg/ml leupeptine, and 1 mmol/l phenylmethanesulphonyl fluoride (PMSF). The homogenate was centrifuged for 10 min at $10^3g$. The supernatant was centrifuged at 25 × $10^3g$ for 30 min at 4°C. The resulting membrane pellet was homogenised in 1 ml of 20 mmol/l Hepes at pH 7.4, 1 mmol/l PMSF, aliquoted, and frozen at −70°C. Protein was quantified by Lowry's assay [19].

*EGFr binding assay*

Twenty seven human brain tumours (14 gliomas, 12 meningiomas and 1 cerebral metastasis) and one normal human brain sample were analysed by this technique. For each assay, 100 μg of membrane preparation were briefly suspended in 50 μl of 20 mM Hepes buffer supplemented with 0.1% and 10 mmol/l $MgCl_2$, and incubated in triplicate with 1 nmol/l ($^{125}$I)-EGF for 1 h at 25°C in the presence or absence of unlabelled EGF (100 nmol/l, duplicate samples). The reaction was stopped by filtration through GF/C glass fibre filters (Whatman) followed by two washes with 5 ml of chilled phosphate buffered saline (PBS) pH 7.2, 0.1% BSA. For each sample, the radioactivity retained on the filter was counted and specific binding was calculated and expressed as fmol EGF bound/mg membrane proteins. Using a chloramine T modified method [20], EGF (Boehringer) was iodinated with Na$^{125}$I (ORIS/CEA) to a specific activity ranging from 200 to 300 μCi/μg (7.4–11.1 MBq) of peptide. Binding to A431 cell membranes was used as a positive control.

*Table 1. Human nervous system tumours*

| Type | Human brain tumour no | Age in years (sex) | Histology WHO classification | Survival |
|---|---|---|---|---|
| Gliomas | G 2 | 17 (M) | Oligo astrocytoma | A (2) |
| grade II | G 3 | 35 (M) | Oligo astrocytoma | D (36) |
| | G 18 † | 35 (M) | Microcystic astrocytoma | |
| | G 34 | 33 (F) | Microcystic astrocytoma | A (7) |
| Gliomas | G 7 † | 27 (M) | Astrocytoma | D (36) |
| Grade III | G 8 | -- (M) | Astrocytoma | |
| | G 10 | 61 (M) | Astrocytoma | D (11) |
| | G 11 | 17 (F) | Astrocytoma | D (13) |
| | G 14 | 40 (M) | Anaplastic astrocytoma | |
| | G 15 | 66 (F) | Astrocytoma | D (5) |
| | G 19 † | 30 (F) | Astrocytoma | |
| | G 35 | 48 (M) | Astrocytoma | D (8) |
| | G 37 | 59 (F) | Astrocytoma | |
| | G 38 | 63 (M) | Astrocytoma | D (4) |
| | G 49 | 44 (F) | Anaplastic astrocytoma | D (11) |
| | G 65 | 50 (F) | Astrocytoma | D (7) |
| Gliomas | G4 | 69 (M) | Glioblastoma | D (48) |
| grade IV | G 5 | 73 (M) | Glioblastoma | |
| | G 13 | 55 (F) | Glioblastoma | D (12) |
| | G 16 | 32 (M) | Glioblastoma | |
| | G 20 | 55 (M) | Glioblastoma | D (30) |
| | G 36 | 50 (M) | Gliosarcoma | D (3) |
| | G 40 | 41 (M) | Glioblastoma | D (1) |
| | G 42 | 54 (F) | Glioblastoma | |
| | G 43 | 69 (F) | Glioblastoma | |
| | G 44 | 69 (M) | Glioblastoma | D (4) |
| | G 48 | 60 (F) | Glioblastoma | D (14) |
| Brain | Met 1 | 65 (M) | Epidermoid carcinoma (lung) | D (60) |
| metastases | Met 23 | 50 (M) | Adenocarcinoma (lung) | D (3.5) |
| | Met 24 | 58 (F) | Epidermoid carcinoma (matrix) | |
| | Met 25 | 62 (M) | Adenocarcinoma (lung) | |
| | Met 28 | 66 (M) | Adenocarcinoma (unknown origin) | D (4) |
| | Met 30 | 45 (F) | From melanoma | D (6) |
| | Met 39 | 55 (M) | Epidermoid carcinoma (lung) | D (10) |
| | Met 41 | 62 (F) | Adenocarcinoma (breast) | D (21) |
| | Met 63 | 59 (F) | Adenocarcinoma (unknown origin) | D (15) |
| | Met 75 | 42 (M) | Malpighian carcinoma (lung) | |
| | Met 88 | | Adenocarcinoma (breast) | |
| Neuroblastomas | Neu 53 | -- (M) | Neuroblastoma | A (4) |
| | Neu 83 | -- (M) | Neuroblastoma | A (0.75) |
| Medulloblastoma | Med 12 † | 39 (F) | Medulloblastoma | D (8) |
| Meningiomas | M 31 | 72 (F) | Meningotheliomatous | A (7) |
| | M 33 † | 74 (F) | Psammomatous | D (12) |
| | M 45 | 70 (F) | Fibroblastic | A (8) |
| | M 55 | 76 (f) | Transitional | A (3) |
| | M 56 | 74 (F) | Fibroblastic | A (4) |
| | M 57 * | 56 (F) | Transitional | A (3) |
| | M 58 | 29 (F) | Meningotheliomatous | A (3) |
| | M 59 | 56 (F) | Meningotheliomatous | D (0.75) |
| | M 60 | 48 (M) | Angioblastic hemangiopericytic | A (9) |
| | M 62 | 39 (M) | Transitionnal | A (9) |
| | M 64 | 33 (F) | Meningotheliomatous | A (2) |
| | M 66 | 59 (M) | Meningotheliomatous | A (1.75) |
| | M 67 | 69 (M) | Transitionnal | |

G: glioma; M: meningioma; Med: medulloblastoma; Neu: neuroblastoma; Met: brain metastasis; D: dead (survival months). A: alive (elapsed time, years).

* Case M 57 was treated with irradiation for scalp-disease 40 years ago and also developed a cancer of the thyroid gland.

† Recurrent cases.

*Table 2. EGFr gene and EGFr binding studies in a series of 27 nervous system tumours*

| Tumours | | EGFr gene Amplification of gene | EGFr gene Expression mRNA | EGFr protein Binding to EGF (fmol/mg) | EGFr protein Mean number of receptors (S.D.) |
|---|---|---|---|---|---|
| Gliomas G II | G 3 | + | + | 23 | |
| | G 34 | + | + | 45 | |
| | G 7 | + | + | 10.5 | |
| Gliomas G III | G 14 | + | + | 12 | |
| | G 15 | +++ | ++++ | 35 | |
| | G 19 | + | + | 3.5 | |
| | G 35 | + | + | 10 | 37 (31) |
| | G 38 | +++ | +++ | 51 | fmol/mg |
| | G 65 | +++ | − | 89.3 | (14 gliomas) |
| Gliomas G IV | G 5 | ++ | +++ | 61 | |
| | G 16 | + | + | 12 | |
| | G 20 | +++ | +++ | - | |
| | G 40 | +++ | +++ | 64 | |
| | G 43 | + | + | 91 | |
| | G 44 | + | + | 6 | |
| Meningiomas | M 31 | + | + | 5.2 | |
| | M 33 | + | + | 10.7 | |
| | M 45 | + | + | 33 | |
| | M 55 | + | + | 13.9 | |
| | M 56 | + | + | 16 | |
| | M 57 | + | + | 48 | 13 (14) |
| | M 58 | + | + | 10.1 | fmol/mg |
| | M 59 | + | + | 5 | (12 meningiomas) |
| | M 60 | + | + | 0.5 | |
| | M 64 | + | + | 12 | |
| | M 66 | + | + | 3.9 | |
| | M 67 | + | + | 0 | |
| Metastasis | Met 63 | + | + | 2.2 | |
| Tumour cell line | A 431 | +++ | ++++ | 2000 | |
| Normal (peritumoural) human brain | N 17 | + | + | 2 | |

Longitudinal study in a series of 27 human nervous system tumours. + to ++++ corresponds to ascending order of EGFr gene amplification or expression.

## RESULTS

*EGFr gene amplification*

EGFr gene amplification was analysed by Southern blot of *Eco*RI DNA digests of 25 gliomas (G), 13 meningiomas (M), 6 metastases (Met), 1 medulloblastoma (Med), and 1 neuroblastoma (Neu). Some results are illustrated in Fig. 1. Fragments of 8.0, 6.8, 5.8, 5.5, 3.6, 2.5, 2.0, 1.8, 1.5 and 1.2 kb were identified in the tumours as in the DNA of A431 cells, in agreement with previous results using this probe [5]. Among the 25 gliomas studied in this technique, 10 (4 grade III and 6 grade IV) presented significant amplification of 5–100 copies per cell (showed by dot blot hybridisation). Moreover, 3 of these 10 gliomas with gene amplification exhibited gene rearrangements as shown by the absence of normal bands at 8 kb for G38 or the presence of an additional band (4.8 kb) for G20 and G40. No EGFr gene amplification was found in the 13 meningiomas nor in cerebral metastases tested for the Met 75. In one meningioma (M45) an additional band (4.8 kb) was found.

The correlation between EGFr gene amplification (Table 2) and patient age or survival (Table 1) was studied. The average age of the 9 patients who exhibited EGFr gene amplification (grade III and IV) was [mean (S.D.)] 57.4 (9.4) years. For 13 patients who presented a glioma (grade III and IV) without EGFr gene amplification the average was 47.3 (17.5) years. The difference between these two categories was not statistically significant.

The survival of patients who presented a glioma (grade III and IV) with EGFr gene amplification ($n = 7$) was on the average 10.4 (9.7) months and 16.8 (16.3) months for patients with a glioma (grade III and IV) without EGFr gene amplification ($n = 8$). Once again there was no statistically significant difference.

*Expression of the EGFr mRNA*

Expression of EGFr mRNA was studied by northern blot in 36 tumours (19 gliomas, 12 meningiomas, 5 metastases, and 1 neuroblastoma). Some results are presented in Fig. 2. Expression of EGFr mRNA in A431 cells was used as a positive control with three major mRNA species: 10 kb, 5.6 kb and an additional 2.9 kb band encoding a truncated receptor form [5].

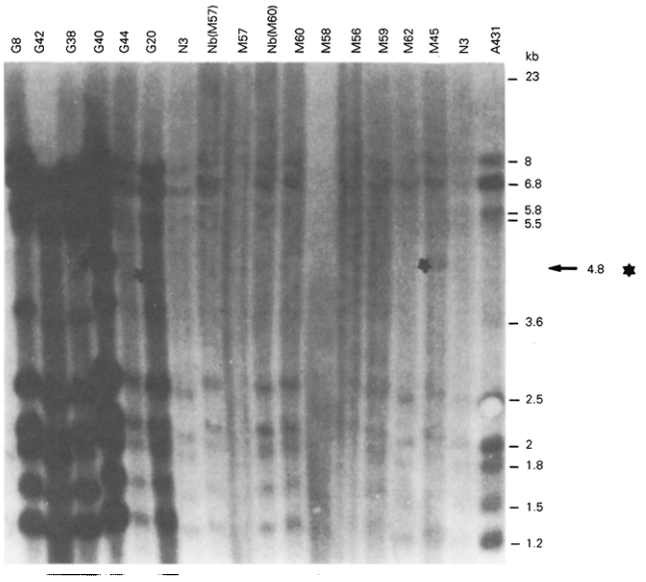


**Fig. 1. EGFr gene amplification in human brain tumours. 20 μg of DNAs from glioma (G), normal (peritumoural) brain tissue (N3), leucocytes of patients with meningioma: normal blood (Nb), meningioma (M), and from A431 cell line used as the control. EGFr gene amplification is observed for the G8, G42, G38, G40, G44 and G20 gliomas, and the A431 cell line. This amplification is associated with a mutation (additional 4.8 kb band) in G40 and G20 gliomas. EGFr gene amplification was not observed in meningiomas. However, an additional 4.8 kb band is found in M45 meningioma. Exposure was for 2 days.**

The 25 non-amplified EGFr DNA tumours had their EGFr mRNAs non-overexpressed. The EGFr mRNAs had the same pattern as in normal brain: a major band at 10 kb, bands at 4.3 kb, 1.8 kb and a minor band at 5.6 kb observed after longer exposure time. From the 19 gliomas and 5 brain metastases studied by northern blot, 9 gliomas and 1 metastasis showed

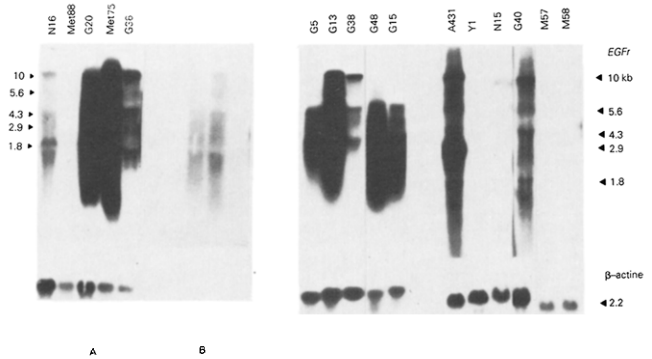


**Fig. 2. Investigation of EGFr mRNA overexpression in human brain tumours. Exposure was for 3 days for all films except for B: same filter as A but exposed for 1 day. Northern blot analysis of total cellular RNA from peritumoural (N15 10 μg, N16 30 μg), tumoural brain tissue 10 μg (G: glioma, Met: metastasis, M: meningioma) and two cell lines [A431 and Y1 (murine adrenocortical tumour cell line) 10 μg] used as positive and negative controls. Successive hybridisations of the same filters have been done with pE7/EGFr and β-actin probes. The pattern of EGFr mRNA is not the same in all tested tumours: the major messenger of 10 kb is found in meningiomas, peritumoural tissue, and in gliomas not exhibiting EGFr gene amplification; in gliomas exhibiting EGFr gene amplification 4.3 kb and 1.8 kb are the major messengers, except in G38 glioma where the 10 kb messenger remains the major one.**

significant overexpresion from 10 to 50-fold (confirmed by dot blot assays) of four types of mRNA at 10, 5.6, 4.3 and 1.8 kb, with differences in their stoechiometry between tumours. The EGFr mRNAs were always overexpressed in the gliomas in which the EGFr gene was found to be amplified (53% of gliomas of grades III and IV). No significant increase in EGFr gene expression was observed in the meningiomas.

*Binding assay*

The binding activity of EGF receptor was investigated in 27 human brain tumours (14 gliomas, 12 meningiomas and 1 cerebral metastasis of adenocarcinoma), and in one peritumoural human brain sample. For each type of tumour and other tissues, the results obtained by this technique are presented in Table 2. Receptor levels varied from 0 to 91 fmol EGF bound/mg of membrane proteins. EGF receptor levels were equal to 13 (14) fmol/mg of membrane proteins for the meningiomas ($n$ = 12), 37 (31) fmol/mg for the gliomas ($n$ = 14) and 2 fmol/mg for the single cerebral metastasis analysed as well as for the peritumoural brain sample.

*Correlation between EGFr levels and EGFr gene amplification (Table 3)*

The difference between EGFr levels from EGFr gene amplified tumours ($n$ = 5) and from all non EGFr gene amplified tumours ($n$ = 22) was statistically significant ($P \leq 0.001$).

The difference between EGFr levels from EGFr gene amplified tumours ($n$ = 5) and from non EGFr gene amplified gliomas ($n$ = 9) ($P = < 0.05$) was also statistically significant, as well as for the meningiomas ($n$ = 12) ($P \leq 0.001$).

## DISCUSSION

27 human brain tumours (14 gliomas, 12 meningiomas, and 1 cerebral metastasis) were tested in a longitudinal study by Southern blot, northern blot, and binding assay techniques. The purpose of this study was first to establish correlations between the grade of the tumour and EGFr gene amplification and EGFr mRNA overexpression (Table 2). In agreement with the results of Wong *et al.* [21] and Libermann *et al.* [9], EGFr mRNA overexpression which is observed in 40% of high grade gliomas is invariably associated with EGFr gene amplification. EGFr gene amplification and EGFr mRNA over-expression are never observed in meningiomas. Only malignant tumours have an activated EGFr gene which may be associated with mutations of this gene (G20, G38, and G40). Activation of the EGFr gene therefore seems to be related to high grade malignancy.

The correlation between EGFr levels and EGFr gene amplification (Table 3) was studied.

The tumours exhibiting EGFr gene amplification and mRNA overexpression always have an increased level of EGF receptors. A relationship with malignancy appears to exist when the two criteria, EGFr gene amplification and high receptor level, are associated. Therefore, amplification of the gene could be a pathway of EGFr gene activation leading to high production of EGF receptors. However, for the glioma G43 the high amounts of EGFr found (91 fmol/mg of membrane proteins) are not the result of EGFr gene amplification, as it has been also reported in numerous tumour cell lines [22, 23]. Therefore, another regulatory pathway of EGFr production, situated at translational and/or post-translational levels, could exist; for example, membrane components recycling may explain an EGFr overexpression in the absence of gene amplification.

A strong deregulation of the *c-erb* B proto-oncogene is initiated

*Table 3. Correlation between the EGF receptor level and EGFr gene amplification*

| EGFr gene | EGFr mRNA | Neoplasias | Grade | *n* | EGF receptor levels (S.D.) (fmol/mg of prot.) |
|---|---|---|---|---|---|
| Amplified | Overexpressed | Gliomas | III | 3 | 58.4 (27.9) |
| | | | IV | 2 | 62.5 (2.1) |
| | | | Total | 5 | 60.1 (19.9) |
| | | Meningiomas | | 12 | 13 (14) |
| Non-amplified | Non-overexpressed | Gliomas | II | 2 | 34 (16) |
| | | | III | 4 | 9 (16) |
| | | | IV | 3 | 36 (47) |
| | | | Total | 9 | 23.7 (28.2) |
| | | Cerebral metastasis | | 1 | 2 |
| | | Peritumoural brain | | 1 | 2 |

Correlation between the EGFr level and EGFr gene amplification.

by an amplification process, leading to unregulated overexpression of its functional product, probably responsible for part of the transformation or tumour progression. Another situation, in which more overproduction of normal EGF receptor exists, could be involved in the transformation process of tumours exhibiting EGF-dependent proliferation. Nevertheless, only 42% (5/12) of the high grade gliomas exhibit an amplified and overexpressed EGFr gene. An additional 8% (1/12) have only an elevated level of EGFr. Therefore, 50% (6/12) of the high grade gliomas do not exhibit any EGFr functional or structural abnormality. Brain tumours, and especially gliomas, are known to be highly heterogeneous. Therefore, both a dilution effect due to contamination by normal tissue persisting in invasive tumours, and heterogeneity, can account for the absence of detectable EGFr activation in these tumours. *In situ* hybridisation with EGFr probe would be an appropriate tool to study these aspects more precisely.

However, EGFr abnormalities (DNA amplification, RNA overexpression, mutations, increases EGFr levels) could be only a consequence rather than a cause of neurooncogenesis. They might still be a cause of transformation if it is demonstrated to be one aspect of a more general process found to be involved in every case of glioma. Furthermore, the EGF receptor is certainly not the only key unlocking the regulation mechanisms which maintain a normal cell in its non-transformed state. Even secondary, the EGFr pathway must be considered with interest for therapeutical target for glioma [24].


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# High Frequency of Tumour Cell Reversion to Non-tumorigenic Phenotype

**Vadim A. Lavrovsky, Marina A. Guvakova and Yan V. Lavrovsky**

**Nine spontaneously transformed cell lines were isolated from embryo fibroblasts of mice and rats with different genotypes. In six cell lines highly tumorigenic cell variants were selected. At the start of culture all cell lines were of low or zero tumorigenicity. The same cells in a confluent monolayer *in vitro* had high contact inhibition of growth and proliferated in response to stimulation by growth factors. Tumour progression of the established lines was accompanied by significant changes of these properties. Clonal analysis of the six most malignant cell lines revealed their capacity to revert simultaneously to the non-tumorigenic state and to their initial growth characteristics. Frequencies of reversion to the non-tumorigenic phenotype were much higher than re-reversion to the tumorigenic phenotype. The reversions occurred in several sequential passages of transformed clones, with some variations in individual clones. These observations suppose that frequencies of tumour reversions are a constant genetic characteristic of every cell line.**
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## INTRODUCTION

THE DISCOVERY of polypeptide growth factors appears to link logically the regulation of proliferation of normal somatic cells and the most serious pathology of these processes, tumorigenesis [1–3]. There are many steps that can be defective in tumour cells, from perception of the growth signal by the cell membrane to realisation of the signal in DNA. The main defects, common to all tumour cells, are hard to detect because of their great variability. There may be no adequate models to describe repeatedly and independently neoplastic transformation compared with spontaneous reversals in cell culture. We propose one such model. Nine independent cell lines of fibroblasts with various genotypes were obtained from mice and rats. The cell lines transformed spontaneously *in vitro*. In six of them we succeeded to select highly tumorigenic variants. All nine lines at the beginning of transformation were found to be not tumorigenic at all or very slightly tumorigenic. At the same time they demonstrated high ability to the density-dependent inhibition of cell growth and possessed sensitivity to some serum growth factors. During the tumour progression both properties changed significantly. Besides that, selected highly tumorigenic cells were shown to be able to reverse to a non-tumorigenic or slightly tumorigenic state. At the same time cells reverse to the initial state regarding to both above-mentioned properties.

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## MATERIALS AND METHODS

### *Cell lines*

Inbred lines of mice, C3H/He, CBA, BALB/c, C57Bl/6, and rats, Wa, from our institute were used. Primary cultures of mouse FC3HO, FCBAO, FBALBO, FBIO and rat FWaO embryo fibroblasts were obtained by trypsinisation of embryos without head and inner organs. Established cell lines were maintained in culture for more than 1.5 years, and every 2–4 months cells were tested for tumorigenicity by inoculation under the dorsal skin of syngeneic animals ($10^6$ cells per animal). The observation period was 4 months. At the same time cells were frozen in liquid nitrogen. Six cell lines were selected for high tumorigenicity by passing them *in vivo*. They then were maintained *in vitro*: FC3H3v7, FC3H4v8, FCBA2v10, FBALB1v12, FB12v12, FWa3v6 (the figure after "v" is the number of passages *in vivo*). Moreover, highly metastatic cell lines, FC3H3m and FCBA2m, were selected from cell lines FC3H3 and FCBA2v40.

Primary cultures and established cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The cells were cultured in plastic dishes (Flow) and were trypsinised with 0.025% trypsin (Serva). The cells were passaged three times per week. All cell cultures were found to be mycoplasma-free [4]. Cell cloning was done by