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Distribution of epidermal growth factor receptor gene amplification in brain tumours and correlation to prognosis

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Abstract In 75 gliomas and 31 meningiomas, mutations at the epidermal growth factor receptor (EGFR) gene locus were restricted to gliomas. The ligands of this receptor, epidermal growth factor and transforming growth factor alpha, lacked quantitative changes at their loci in gliomas and meningiomas. EGFR gene amplification occurred in astrocytomas, oligodendrogliomas, ependymomas and glioblastomas. The frequency of this mutation significantly increased with the malignancy grade and the patient's age. Especially in glioblastomas of individuals aged over 64 years, EGFR gene mutations were observed without chromosome-10-specific allele losses. This finding contradicts the hypothesis that deletion of one entire chromosome 10 regularly precedes EGFR gene amplification in primary glioblastomas of patients aged over 50 years. It was found that most individuals whose gliomas carry an EGFR gene mutation have a poor prognosis, comparable to that of glioblastoma patients even when the tumour is graded as benign.

Key words EGFR gene amplification and expression · Brain tumours · Biological factors · Prognosis

Introduction

Malignant progression of gliomas is accompanied by epidermal growth factore receptor (EGFR) gene amplification [13], which occurs in some 1% of benign tumours and 35% of glioblastomas. In addition, 40% of these amplified DNA sequences are rearranged. EGFR gene amplification is said to be a non-morphological parameter for malignancy in gliomas, but most studies lack clinical data. Only Bigner et al. [1] and Hurtt et al. [9] compared clinical courses to EGFR gene mutations in glioblastomas, and they failed to demonstrate prognostic significance.

The amplification of the EGFR gene leads to enhanced expression of the receptor. Raised expression of EGFR also occurs in meningiomas, but overall it does not reach the same level as in gliomas. Only a few meningiomas have been studied for EGFR gene mutations, and no amplification of DNA sequences could be detected.

The ligands of EGFR, epidermal growth factor (EGF) and transforming growth factor alpha (TGFα), are expressed in most gliomas [8]. Yung and coworkers [19] reported amplification of TGFα gene sequences in astrocytomas as a cause for enhanced expression.

The aim of this survey was to study the distribution of gene amplification at the EGFR, EGF and TGFα loci in gliomas and meningiomas. Further, the clinical data were analysed for significant differences in relation to EGFR gene amplification.

Material and methods

Histological diagnosis, malignancy grade, age, sex and clinical course were considered for evaluation. Regrowth of a tumour had to be confirmed by computed tomography. Subsets of gliomas were analysed for loss of alleles at chromosome-10-specific loci [2, 3] and EGFR expression. Histological diagnosis and malignancy grading were according to the WHO classification [11].

Table 1 cDNA probes for hybridizations. Chromosomal location, Locus, name of the probe, restriction enzymes and the fragment lengths after restriction enzyme digest are listed. The origin of the probes is given as references (Ref). The probes for TGFa and EGF loci were ordered from ATCC (USA)

Chromosomal location	Locus	Probe	Restriction enzyme	Fragment lengths (kb)	Ref.
1p13–12	EGFR	pE7	*Hin*dIII	16.5/2.7 (constant) 12.5/10; 5.4/5.1 (RFLP)	[18]
2p13	TGFa	phTGF1-10-3350	*Bam*HI	4/7/11 (RFLP)	[14]
4q25–q27	EGF	pHEGF121	*Hin*dIII	1.9	[15]

Table 2 Patients' age and survival time in relation to EGFR gene mutations, histological diagnosis and malignancy grade in gliomas and meningiomas. Survival times are given in parantheses when there was no regrowth of the tumour or the patients were still alive. + Present; – not present; *n* number of patients, *pil.* pilocytic, *As.* astrocytoma, *Ep.* ependymoma,, *Ol.* oligodendroglioma *Glb.* glioblastoma, *Men.* meningioma

Grade (WHO)	Diagnosis (WHO)	EGFR gene	*n*	Age (years)		Survival time without progression of tumor (month)		Survival time (month)	
				Median	Range	Median	Range	Median	Range
I	pil. As	+	0						
		–	3	18.7	6 – 45	(32)[a]	(24) – (48)	(32)[a]	(24) – (48)
I	Ep.	+	0						
		–	4	18.5	7 – 31	(28)[a]	(3) – (48)	(28)	(3) – (48)
II	As.	+	1	58		3		6	
		–	4	39.3	26 – 66	48	18 – (50)	(41)[b]	(33) – (50)
II	Ep.	+	0						
		–	4	20.7	5 – 41	> 58[c]	3 – (24)	>10[c]	5 – (35)[c]
II	Ol.	+	1	61		3		4	
		–	2	51.5	34, 69	–[d]	(12), 36	–[d]	(12), 38
III	As.	+	2	28	7, 49	8.5	3, 14	17.5	21, 14
		–	6	45.2	26 – 67	17.5	2 – (48)	18.5	2 – (48)
III	EP.	+	1	50		5		5	
		–	4	24	2 – 49	10	3 – 22	19.5	10 – 44
III	Ol.	+	5	64.6	60 – 72	6	3 – 7	8.5	6 – (24)
		–	6	45.2	26 – 65	11	1 – 48	14	1 – (60)
IV	Glb.	+	13	63.4	53 – 76	4	3 –16	7	3 – 22
		–	19	62.7	26 – 78	5	1 – 12	6	1 – 15
I	Men.	+	0						
		–	21	53.1	29 – 71	> 54[e]	13 – (108)	(60)[f]	24 – (108)
II	Men.	+	0						
		–	3	51.3	38 – 63	(55)[a]	(48) – (64)	(55)[a]	(53) – (64)
III	Men.	+	0						
		–	7	64	46 – 76	23	4 – (47)	> 49[g]	(14) – (83)

[a] Median follow-up, all patients alive without regrowth of tumour
[b] Median follow-up, all patients alive
[c] One patient had regrowth of the tumour after three months and was still alive after 35 months, one patient died after five months
[d] No median values given
[e] Median follow-up, in six cases regrowth of the tumours after 13 to 96 months
[f] One patient died from cardiac disease after 48 months without regrowth of meningioma
[g] Two patients died after 46 and 49 months

We prepared genomic DNA from the tumour tissue after treatment with proteinase K. White blood cell (WBC) DNA was obtained as described previously [6, 7]. DNA was digested with a 10-fold or higher excess of restriction enzymes. Samples containing 8 µg genomic DNA were run on 0.8% agarose gels. DNA fragments were transferred from the gels to nitrocellulose filters and fixed by baking in a vacuum. The filters were prehybridized in a 5 × SSC (1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate)–5 × Denhardt solution at 68°C for 2 h and hybridized in a 5 × SSC–5 × Denhardt solution, plus 10% dextran sulphate, with a 5 ng/ml radioactively labelled probe for 4 h at 68°C in the presence of a 0.2 mg/ml sonicated, denatured salmon sperm competitor DNA. Re-

combinant plasmids labelled with [alpha-^{32}P]dCTP were used as probes. After hybridization the filters were washed in 0.1 × SSC–0.1% SDS (sodium dodecyl sulphate) and subsequently in 0.1 × SSC, both at 63°C. Filters were autoradiographed for 18–72 h at –70°C with two intensifying screens. The probes for hybridizations are listed in Table 1.

The grade of EGFR gene amplification was determined by different procedures. Tumour DNA and WBC DNA were loaded in identical concentrations in adjacent slots on agarose gels. The intensities of bands were compared by laser scanning. In those tumours which were heterozygous for a *Hin*dIII polymorphism, direct comparison of the intensities of the heterozygous bands was possible within one lane. Tumour DNAs were diluted and analysed according to their grade of amplification. Further, the intensities of bands were compared after double hybridization with pE7 and probes of other chromosomes. DNAs with a known grade of EGFR amplification were available for use as standards for each gel. In positive cases the grade of EGFR gene amplification in tumour DNA was at least five times that of the diploid genome.

The expression of the EGFR was estimated by binding the monoclonal antibody (Mab) 425 [16], which is an IgG2a antibody specific for a polypeptide epitope of the human EGFR. After resection the tumour material was immediately frozen in isopentane, cooled in liquid nitrogen and then kept at –80°C until the tissues were cut into 10-µm-thick sections at –20°C in a cryostat. The sections were fixed in cold acetone (4°C) for 5 min and were kept at –20°C overnight for up to 5 days. For avidin-biotin-coupled immunoperoxidase staining the slides were incubated with Mab 425 for 60 min, with biotinylated horse antimouse IgG for 20 min and with avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, Calif.) for 20 min. All incubations were done in a moist chamber. The washes after incubation consisted of one flush and two 5-min baths with phosphate buffered saline. After the last wash the sections were treated for 5 min with freshly prepared 3-amino-9-ethyl-carbazole as chromogen and counterstained with haematoxylin. Then the slides were mounted in Mowiol (Hoechst, Germany).

Statistical analyses were carried out by the computer program SAS (Cary, N.C.).

Results

EGFR gene mutations were found in 23 of 75 gliomas. These EGFR gene mutations were amplifications of DNA sequences and, in three gliomas, rearrangements at the EGFR locus (in two cases without obvious amplification of DNA sequences). EGFR gene mutations were not detected in 31 meningiomas. Within the group of gliomas, the EGFR gene mutations were not restricted to any histological entity (Table 2). The overall frequency of EGFR gene mutations in gliomas was 31%, with a significant increase from 0% in grade I tumours to 41% in glioblastomas ($P = 0.017$, Mantel-Haenszel test; see also Fig. 1). The patients whose gliomas carried an EGFR gene amplification had an average age of 59.4 years (only one was under 49 years), compared with an average age of 44.7 years ($P = 0.0117$, Mann-Whitney test; see also Fig. 1) for those whose gliomas lacked an EGFR gene mutation.

The sex of the patient and the duration of preoperative symptoms were not associated with a higher incidence of EGFR gene mutations. The results of the survival time and age analyses are listed in Tables 2 and 3, and examples are shown in Fig. 2. In grade I–III gliomas with EGFR

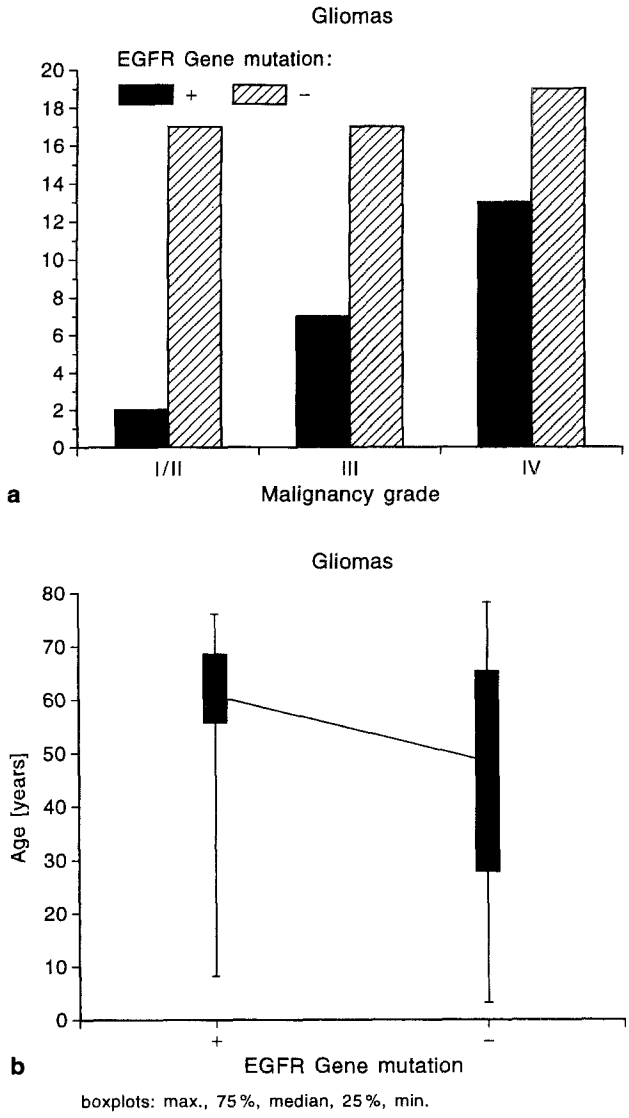

Fig. 1 **a** Frequency block chart of EGFR gene mutations in relation to malignancy grades in gliomas; **b** age distribution of all glioma patients with and without EGFR gene mutations. For number of cases see Table 2

gene mutations the median survival time ranged from 5 to 9 months, in the range of glioblastoma patients. In the glioblastoma group there were no significant prognostic differences between patients with and without EGFR gene mutations.

EGF and TGFα loci lacked amplification and rearrangements of DNA sequences in gliomas and meningiomas. The TGFa locus was analysed in 6 grade I/II gliomas, 5 grade III/IV gliomas, 10 grade I/II meningiomas and 1 grade III meningioma. The EGF locus was analysed in 6 grade I/II gliomas, 5 grade III/IV gliomas, 8 grade I/II meningiomas and 1 grade III meningiomas.

Loss of chromosome 10 alleles was found in none of 7 grade I/II gliomas, in 2 of 14 grade III gliomas (1 astrocy-

Table 3 Median survival times and median patient age in relation to EGFR gene mutations and malignancy grade of gliomas

Grade (WHO)	EGFR gene mutation	*n*	Median age (years)	Median survival time without progression of tumour (month)	Median survival time (month)
I – IV	+	23	59.4	5[a]	6[b]
	–	52	44.7	11	12
I/II	+	2	59.5	3	5
	–	17	27.8	> 36	> 38
III	+	8	53.6	5.5[c]	9
	–	16	39.9	11	14
IV	+	13	63.4	4	7
	–	19	62.7	5	6

P-values of significant differences (log-rank test): [a] 0.0024, [b] 0.0046, [c] 0.0380

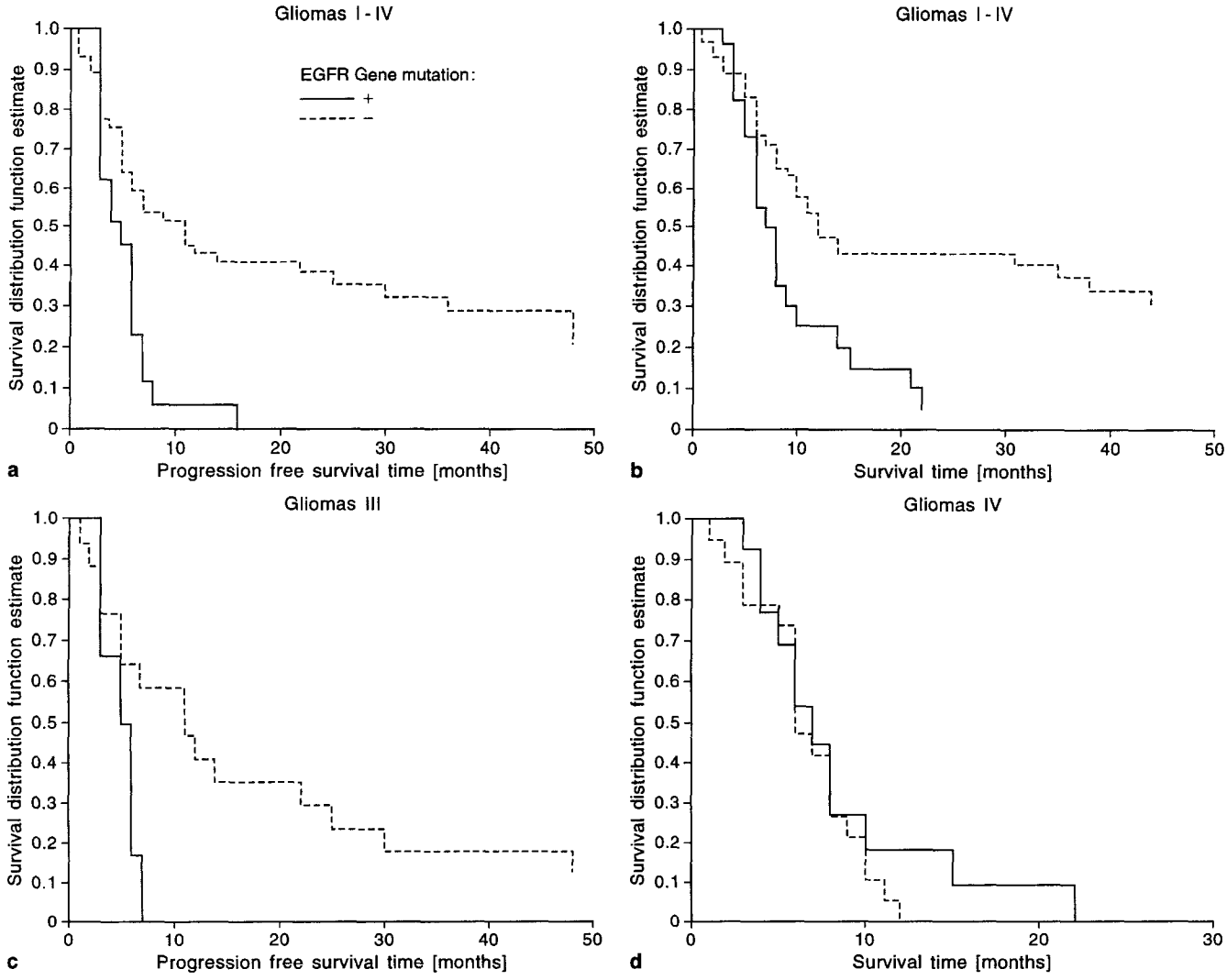

Fig. 2a–d Survival time curves of patients with gliomas in relation to grades of malignancy with or without EGFR gene mutations; see also Table 3. EGFR gene mutations were present in 23 cases and absent in 52 cases

toma, 1 oligodendroglioma) and in 5 of 15 glioblastomas (2 glioblastomas with EGFR gene amplification and 3 glioblastomas without EGFR gene mutations). EGFR gene amplification was not associated with loss of chromosome 10 alleles in any glioblastomas of patients over 64 years old (three cases, in which both kinds of mutations were analysed).

Increased EGFR expression, immunohistochemically detectable by Mab 425, was present in 2 of 10 grade I/II gliomas (1 ependymoma, 1 astrocytoma), 11 of 17 grade III gliomas (3 of 6 astrocytomas, 1 of 3 ependymomas, 7 of 8 oligodendrogliomas),19 of 22 glioblastomas, 6 of 9 grade I/II meningiomas and 5 of 6 grade III meningiomas. In 13 of 14 gliomas with EGFR gene amplification the EGFR expression was enhanced.

Discussion

Within the most common human brain tumours, EGFR gene amplification is restricted to gliomas. Meningiomas lack this kind of mutation even when the EGFR is strongly expressed in malignant tumours. EGFR gene mutations occur independently of histological subclassification in glioblastomas, astrocytomas, oligodendrogliomas and ependymomas. The frequency of these mutations increases with the malignancy grade of gliomas and the age of the patients (Fig. 1). All patients (except one with a glioblastoma) whose gliomas carried this mutation had a very short progression-free survival time. On the other hand, it must be acknowledged that our data are not age-corrected. However, even younger patients (e.g. one 7-year-old patient with regrowth of an astrocytoma after 3 months) had only a short period until progression of the tumour, in the range of patients with glioblastomas. So this survey shows that EGFR gene amplification was associated with a poor prognosis concerning tumour regrowth in grade I–III gliomas. In glioblastomas the occurrence of EGFR gene mutations was not associated with a worse prognosis. At the DNA level no changes could be found at the loci of the EGFR ligands, EGF and TGFα, in gliomas or mengiomas, so the previously reported amplification at the TGFa locus [19] does not seem to be a frequent event in gliomas.

EGFR gene amplification is said to require preceding loss of chromosome 10 in primary glioblastomas [4, 5, 12] of older patients. In our series of glioblastomas there were some patients whose tumours lacked loss of chromosome 10 in the presence of EGFR gene mutations. Compared with those reports in which age is given [5, 10, 17], we have studied a high proportion of patients over 64 years of age. This subgroup often seems to lack the loss of the entire chromosome 10. Furthermore, in our grade III gliomas EGFR gene mutations occurred without chromosome 10 loss. It can therefore be assumed that although there is high association of EGFR gene amplification with chromosome 10 losses in glioblastoma patients between 50 and 60 years of age, in older patients loss of chromosome 10 is not a precondition for such amplification.

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