Prognostic implications of p53 protein, epidermal growth factor receptor, and Ki-67 labelling in brain tumours

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Summary The expression of p53 protein, epidermal growth factor receptor (EGFR), and Ki-67 nuclear antigen was examined by immunohistochemistry in biopsies of 16 types of human brain tumours, including 43 astrocytomas. P53 protein, almost certainly its mutant form, was expressed in seven of the 16, and EGFR in 11 of the 16 types of tumours. In astrocytomas both the proportion of tumours which expressed p53 or EGFR increased with grade of malignancy as did the mean Ki-67 labelleing index (LI): p53–0% in grade 1, 17% in grade 2, 38% in grade 3, 65% in grade 4; EGFR–0% in grade 1, 33% in grade 2, 85% in grade 3, 95% in grade 4; mean Ki-67 LI–1.1% in grades 1 and 2, 8.3% in grade 3, and 13.4% in grade 4. Astrocytomas which expressed p53 or EGFR had a significantly higher Ki-67 LI at $P < 0.05$ (11.8% and 10.7%, resp.) than those that did not (6.2% or 4.1%, resp.). Patients with astrocytomas expressing p53 or EGFR had a significantly reduced survival ($P = 0.035$ and $P = 0.007$, resp.): only 11% of the p53 + ve and 13% of the EGFR + ve patients were alive at 100 weeks following diagnosis compared to 36% of p53-ve or 60% of EGFR-ve patients. Patients with Ki-67 LI > 5% had a reduced survival ($P < 0.0001$) – none survived beyond 86 weeks following diagnosis, whilst 63% of patients with < 5% positive cells were still alive at 100 weeks. The univariate analysis showed that in astrocytomas expression of p53 mutants, EGFR protein, and Ki-67 > 5% are associated with malignant progression and poor prognosis. The multivariate analysis revealed that only tumour grade and Ki-67LI were independent prognostic factors for survival.

Glial tumours are the most common primary tumours of the CNS (Russell & Rubinstein, 1989). Almost all types of glial tumours can recur and display malignant progression to some degree depending on the histopathological type of tumour, grade of malignancy, its location, the patient's age, and the extent of surgical resection (Russell & Rubinstein, 1989). However, the onset of the malignant process is highly variable, and prognostic predictions cannot be made in individual patients. In both low and high grade astrocytomas loss of heterozygosity for alleles on chromosome 17p has recently been found, suggesting that during early stages of tumorigenesis mutation and acquisition of homozygosity has occurred in a recessive oncogene on that chromosome (James et al., 1989; El-Azouzi et al., 1989). Also, several high grade astrocytomas have been found to contain point mutations in gene p53, which is localised on chromosome 17p (Nigro et al., 1989). Malignant gliomas have also been shown to have abnormal chromosomes 1, 6, 9, 10, 13, 22, sex chromosomes, and an extra chromosome 7 (Bigner et al., 1984; James et al., 1988). The epidermal growth factor receptor (EGFR) gene which is located on chromosome 7 (Shimizu et al., 1985) has been shown to be amplified and rearranged (Liberman et al., 1984; Liberman et al., 1985; Wong et al., 1987; Sugawa et al., 1990), and the EGFR protein found overexpressed in the most malignant gliomas, especially the glioblastoma multiforme (Arita et al., 1989; Reifenberger et al., 1989. On the basis of these findings Bigner and Vogelstein (1990) have proposed a model for malignant progression of gliomas in which losses of chromosomes 17p, 13, or 22 occur in low grade gliomas, and loss of chromosome 10 represents a critical step in transition from grade 3 (anaplastic astrocytomas) to grade 4 (glioblastoma multiforme), whilst abnormalities of 9p and EGFR amplification stimulate further progression. In Primitive Neuroectodermal Tumours (PNETs) chromosomes 1 and 17 have been implicated in tumour

development by cytogenetic studies (Bigner et al., 1988; Griffin et al., 1988), and subsequently allele loss has been found on chromosomes 17p, 6q, and 16q (Thomas & Raffel, 1991). The genes affected by putative mutations on these chromosomes have not yet been identified in PNETs but gene p53 on chromosome 17p is a candidate.

Normal p53 gene behaves as a tumour suppressor gene. It encodes a 53kD nuclear phosphoprotein, which is thought to be involved in regulation of cell growth (Finlay et al., 1989; Stanbridge, 1990). The normal p53 protein is undetectable by standard immunohistochemistry because of its low cellular levels and a very short half-life, about 20 min (Finlay et al., 1989). Point mutations in the gene lead to expression of nonfunctional mutant forms with substantially longer half-lives (up to about 24 h), and an elevation of cellular levels to 10–100 fold above normal values (Finlay et al., 1989) which can be detected by immunohistochemistry (Cattoretti et al., 1988; Iggo et al., 1990; Rodrigues et al., 1990). An association between expression of p53 mutants, epidermal growth factor receptor (EGFR), and poor prognosis has recently been reported to occur in human breast carcinomas (Harris et al., 1990) but has not yet been examined in astrocytomas. EGFR is a 170 kD transmembrane glycoprotein with an extracellular ligand-binding domain, a transmembrane region and an intracellular portion with tyrosine kinase activity (Hunter, 1984). Binding of EGFR or transforming growth factor alfa (TGF-α) to EGFR results in activation of tyrosine kinase activity and stimulation of DNA synthesis, leading to mitosis (Stoscheck & King, 1986; Carpenter, 1987).

The aim of this study was to increase our understanding of basic biological mechanisms in central nervous system (CNS) tumours and to correlate expression of mutant p53 protein and EGFR with tumour proliferative activity and the patients' progress. 78 CNS tumours were examined, including 43 astrocytomas and 6 PNETs using immunohistochemistry and monoclonal antibodies to p53 and EGFR proteins. Monoclonal antibodies to the growth fraction-associated Ki-67 antigen (Gerdes et al., 1984) have been used to assess the proliferative activity of tumour cells as the Ki-67 labelling index (LI) has been shown to correlate with the degree of

malignancy in different types of tumours, including gliomas (Zuber *et al.*, 1988; Raghavan *et al.*, 1990). Preliminary results of this study have been presented in part to the British Neurooncology Society (Jaros *et al.*, 1991*a*) and to the British Neuropathological Society (Jaros *et al.*, 1991*b*).

Materials and methods

Fresh specimens of 78 CNS tumours were obtained during neurosurgery in Newcastle General Hospital between April 1988 and May 1990. They were divided into several portions: (i) used for karyotyping employing methods developed for solid tumours (Adam *et al.*, in preparation); (ii) fixed in formalin, embedded in paraffin, and sections stained with haematoxylin and eosin for histopathological assessment (iii) snap-frozen in arcton pre-cooled with liquid nitrogen and stored at −70°C for immunohistochemistry. Histopathological assessment of the tumours was made according to the WHO classification system (Rorke *et al.*, 1985), and the degree of malignancy was graded according to Kernohan's system (Kernohan *et al.*, 1949).

Frozen sections of the tumours were cut at 6 μm, mounted on silanized glass slides and allowed to dry overnight. The sections were fixed in 1:1 mixture of chloroform:acetone for 10 min at room temperature, dried for 10 min, and incubated with mouse monoclonal antibodies to human p53 (PAb 1801 from Cambridge Research Biochemicals) at a 1:1600 dilution (titred to detect p53 protein expressed in control human lung carcinoma material); or a mouse monoclonal Ki-67 antibodies (Dako) at a 1:25 dilution; or with mouse monoclonal antibodies to EGFR (EGFR1 from Amersham) at a 1:50 dilution (titred on normal human skin), followed by biotinylated anti-mouse antibodies (Vectastain) at a 1:200 dilution, streptavidin-biotin-HRP (Amersham) at a 1:100 dilution, DAB at 0.5 mg ml⁻¹ and counterstained with haematoxylin. All the dilutions of antibodies were prepared in Tris-buffered saline, pH 7.6, containing 1.5% normal preimmune horse serum. As a control for endogenous peroxidase the primary antibodies were omitted on serial sections from each block. Another serial section was stained with haematoxylin and eosin for histopathology.

Immunohistochemically processed sections from all the tumours were examined and those containing nuclei labelled with the p53 or the Ki-67 antibody were classed as p53 + ve (p53 positive) or Ki-67 + ve tumours, and were quantified on a Nikon microscope at × 400 magnification using a square graticule. When regional heterogeneity of labelling was detected in the tumour, counting areas were chosen to include areas with high and low density of p53 positive cells and also areas in which serial sections showed variation in the Ki-67 or the EGFR labelling. In each area between 901 and and 1566 tumour cells were counted from systematically randomised fields. Endothelial cells were not included in the counts, even when in some of the tumours they were labelled with Ki-67 (though never with the p53) antibody. The p53 or Ki-67 LI was calculated as a percentage of labelled tumour cells out of the total number of tumour cells counted (LI = 100 × number of labelled nuclei ÷ total number of nuclei). The highest Ki-67 LI detected in individual tumours was considered to represent the proliferative potential within the tumour (Raghavan *et al.*, 1990), and was therefore used in quantitative analysis. Because the EGFR labelling of tumours varied both in terms of cellular intensity and the proportion of tumour labelled, calculating a simple EGFR LI may have been inadequate. Instead, for each tumour the labelling intensity was scored on a scale from − to + + + +, and the percentage of EGFR reactive tumour cells was calculated using an eyepiece with squared graticule. EGFR labelling factor was then calculated for each tumour by multiplying percentage of the labelled area by 1 if the labelling intensity was scored as − (negative), and by 2,3,4, or 5, respectively, if the labelling intensity was scored as +, + +, + + + or + + + +. Student *t*-test was used to analyse p53, EGFR or Ki-67 labelling as continuous vari-

ables in relation to tumour grade, and to analyse Ki-67 labelling in relation to p53 or EGFR labelling as categorised variables. Pearson's correlation coefficient (r) was used to determine the strength of association between the continuous variables Ki-67 LI, p53 LI, EGFR labelling factor, and patient's age.

The prognostic importance of each of the variables with natural categorisation, i.e. sex, histological grade (1, 2 *vs* 3, 4), p53 (+ ve *vs* − ve), EGFR (+ ve *vs* − ve), extent of surgery (total, subtotal, partial, biopsy), radiotherapy (Y/N), and chemotherapy (Y/N) was assessed using Log-Rank test (Peto *et al.*, 1977). The continuous variables, i.e. age and Ki-67 LI were separately entered into the Cox regression model (Cox, 1972) to yield relative risks and *P*-values. This avoids the need of possibly 'data-driven' categorisation of the variables although Ki-67 was also considered in the form of < 5% *vs* > 5% and analysed using the Log-Rank test. All variables apart from sex, radiotherapy, and chemotherapy were entered into the multivariate analysis. The multivariate analysis was performed by using a forward stepwise application of Cox's Regression model via the BMDP statistical package (Program 2). Variables selected as statistically significant by this procedure had *P* < 0.10. 95% confidence intervals for the relative risks in the multivariate procedure are given by $e^{coeff \pm 1.96SE(coeff)}$.

Results

Clinical histopathological data

These data are summarised in columns 1–5, and 10–13 of Table I. Only the astrocytoma group was sufficiently large to make the Log-Rank test of survival possible. The range of follow-up time was 0–180 weeks (median = 39 weeks). All except one of the patients who are still alive (patient number 276) have reached the 100 weeks follow-up (Table I). Out of the total of 43 astrocytoma patients, three patients who died of other causes were not included in the Log-Rank analysis. The length of patient's survival was significantly related to their age at diagnosis (younger patients showing longer survival: *P* = 0.0002; Table II), and to the histopathological grade of their tumour (*P* < 0.0001; Table III and Figure 1). In Figure 1 note that patients with malignant grades had a significantly reduced survival time – only 6% of patients with grade 3 and 4 were alive at 100 weeks following diagnosis compared to 89% of patients with grade 1 and 2 tumours. Sex of the astrocytoma patients, radiotherapy, chemotherapy or surgery did not significantly affect their survival (Table III), though surgery was weakly significant (*P* = 0.06).

Cytogenetic data

These data are shown in column 9 of Table I. Cytogenetic analysis was performed on short-term cultures of 74% of the tumours; 50% of the tumours were successfully karyotyped. Chromosomal abnormalities were found in 21% of all the tumours: in 13 astrocytomas grade 3 and 4, one angioglioma grade 4, one malignant choroid plexus papilloma, and one metastatic melanoma. In the astrocytomas and the angioglioma the most common abnormality was aneuploidy of sex chromosomes, in particular loss of chromosome Y. Trisomy of chromosome 7, where EGFR gene is known to be localised, was found in one astrocytoma only (patient No. 113). None of the patients were found to have gross rearrangements or deletions of chromosome 17p, where p53 gene is localised.

Immunohistochemistry of p53

No labelling was detected in normal neocortical (12 cases) and normal cerebellar (four cases) tissue adjacent to the tumours. The p53 labelling of tumours was restricted to the tumour cell nuclei. No cytoplasmic labelling was observed, and no endothelial cells were labelled, even in areas showing

Table I Summary of clinical, histopathological, immunohistochemical, and cytogenetic data

1	2	3	4	5	6	7	8		9	10	11	12	13
						Immunohistochemical analysis					Treatment Therapy		Survival
Patient number	Sex	Age yrs	Site of tumour	Histological diagnosis and grade (1–4)	p53 LI %	EGFR labelling intensity − to ++++	area %	Ki67 LI %	Cytogenetic analysis	Surgery	Radio	Chemo	time PostOp Weeks
183	M	18	Cerb Mid	Astro 1 cystic	0.0	−	100	1.0	Normal	Total exc	+	−	129 alive
188	M	15	Cerb	Astro 1 cystic	0.0	−	100	1.2	Normal	Total exc	−	−	2 other causes
217	F	7	Cerb Hemi	Astro 1 cystic	0.0	−	100	0.5	Normal	Total exc	−	−	115 alive
73	M	63	Left Front Pariet Lobe	Astro 2	0.0	−	100	0.1	Not Done	Subtot exc	+	−	178 alive
82	F	19/12	Cerb Hemi	Astro 2	0.0	−	100	2.3	Normal	Subtot exc	−	−	175 alive
104	M	16	Right Lat Ventr	Astro 2 Tuber scl	0.0	+	100	0.0	Failed	Total exc	+	−	162 alive
138	F	4	Cerb	Astro 2 cystic	0.0	−	100	0.4	Normal	Total exc	−	−	152 alive
236	M	24	Left Temp Lobe	Astro 2	2.8	− / +	99 / 1	0.1	Failed	Subtot exc	+	−	107 alive
267	M	4	HypoThal PitFos 3rd Ventr	Astro 2 Recur	0.0	−	100	2.3	Not Done	Subtot exc	−	−	65
276	M	5/52	Post Fos	Astro 2	0.0	−	100	3.1	Failed	Biopsy	−	+	92 alive
60	M	68	Left Pariet Temp Lobe	Astro 3 cystic	0.0	− / + / +++	20 / 50 / 30	7.5	Not Done	Part exc	−	−	11
67	M	47	Right Front Lobe	Astro 3	0.0	+/++ / +++	100	0.1	Not Done	Part exc	+	−	180 alive
126	M	30	Right Temp Lobe	Astro 3	29.4	− / +/++	80–90 / 10–20	3.4	46XY/ 46XY t(1;14)(p22;p12)	Subtot exc	+	+	113
147	F	37	Left Lat Temp Lobe	Astro 3	22.0	+/++++ / +/++++	100 / 100	1.7	Normal	Part exc	−	−	1 other cause
154	F	63	Left Temp Pariet Lobe	Astro 3	1.2	−	100 / 100	1.4 / 7.7	Not Done	Part exc	−	−	9
167	F	44	Left Front Pariet Lobe	Astro 3	15.2	++/++++ / +++/+++++	100 / 100	9.4 / 36.7	Not Done	Biopsy	−	−	2
194	M	61	Left Front Lobe	Astro 3	0.0	− / +/++ / +++/++++	50 / 50	2.3	Normal	Biopsy	−	−	2
203	M	42	Left Post Pariet Occip Lobe	Astro 3	2.2	+++/++++ / +++/++++	100 / 100	5.7 / 9.3	Normal	Subtot exc	+	−	65
207	M	52	Right Front Pariet Lobe	Astro 3	0.0	+/++++	100	11.5	Failed	Part exc	+	−	39
245	M	52	Left Temp Pariet Lobe	Astro 3	0.0	+++/++++ / +++/++++	90 / 10	12.9	45X-Y/46XY	Biopsy	+	+?	26
257	F	44	Right Temp Lobe	Astro 3	0.0	++/++++	85 / 15	2.1	Failed	Part exc	+	−	53
273	M	33	Cerb Hemi	Astro 3	1.6	− / +	>99 / <1	4.5 / 7.5	45X-Y/46XY	Part exc	−	−	9
285	M	12	Right Pariet Lobe	Astro 3	0.0	−	100	3.1	46XY/46XY (16) = ?del 16p	Subtot exc	+	+	77

Table I (continued)

1	2	3	4	5	6	7		8	9	10	11	12	13
						Immunohistochemical analysis EGFR				Treatment			Survival
Patient number	Sex	Age yrs	Site of tumour	Histological diagnosis and grade (1–4)	p53 LI %	labelling intensity – to ++++	area %	Ki67 LI %	Cytogenetic analysis	Surgery	Therapy Radio	Therapy Chemo	time PostOp Weeks
69	M	35	Right Temp Lobe	Astro 4 Recur	19.7	++/+++	100	13.8	Not Done	Part exc	–	+	47
75	M	54	Right Pariet Lobe	Astro 4	0.0	++/+++	100	21.0	Not Done	Biopsy	+	–	48
112	M	33	Left Front Lobe	GlioMulti 4	14.3 15.7	– +	99 1	1.5 3.9	45X-Y/46XY/ 47XXY/47XYY	Subtot exc	+	–	25
113	M	65	Right Occip Lobe	Astro 4	0.0	++/+++++	100	15.3	45X-Y/ 46X-Y + 7/46XY	Subtot exc	–	–	3 other causes
116	F	58	Right Temp Pariet Lobe	GlioMulti 4 cystic	0.0	+++/+++++	100	11.0	Normal	Part exc	+	–	65
134	F	59	Right Temp Lobe	Astro 4	3.5	++/+++	100	14.4	47XX9p+ + 10q- – 12 21 + 19 + 20 + mar	Subtot exc	+	–	37
139	M	44	Left Frong Lobe	GlioMulti 4	0.0	–/+ +++	80–85 15–20	8.1	Not Done	Total exc	+	–	24
142	F	41	Right Temp Lobe	GlioMulti 4	3.2 0.0	+++/+ +++/+	100 100	5.1 11.2	Not Done	Part exc	+	+	50
150	M	37	Right Pariet Temp Lobe Thal Int Caps	GlioMulti 4	15.5	+++/+ – +	100 50 50	7.8	45X-Y/46XY/ 47XXY	Part exc	–?	–?	19
158	M	49	Left Post Pariet Lobe	Astro 4	0.0 0.0	++/+++ +++/+	100 100	13.4 6.3	45X-Y/46XY	Subtot exc	+	–	·51
165	F	50	Right Front Lobe	GlioMulti 4	61.9	++/+++	100	16.7	46XX/46XX t(2;8) (q33;q4?1)/ Hyperdiploid with double minutes	Subtot exc	–	–	37
169	M	67	Left Temp Pariet Lobe	Astro 4	0.0	+++/+++++	100	0.6	45X-Y/46XY	Subtot exc	+	+	24
170	F	55	Right Temp Lobe	GlioMulti 4 cystic	5.6	+/+++	100	13.3	Normal	Part exc	+	–	86
212	F	55	Right Pariet Lobe	Astro 4	4.7 0.5	+/– – +	100 85–90 10–15	8.9 1.5	Not Done	Subtot exc	–	–	5
238	M	67	Right Occip Pariet Lobe	Astro 4	0.3	+++ ++++	99 1	5.1	Failed	Part exc	+	–	40
252	M	60	Right Front Lobe	Astro 4	0.0	++++	100	20.3	Failed	Subtot exc	+	–	35
258	M	68	Right Pariet Occip Lobe	GlioMulti 4	10.5 11.8	+++/+++ +++/+++	100 100	6.7 3.8	45X-Y/46XY	Subtot exc	–	–	17
263	F	45	Left Amyg Thal Hypothal Pedun Pit	Astro 4	0.1 2.1	– +/+++	90–95 5–10	4.5 23.5	Not Done	No Surgery	–	–	0
270	M	59	Right Temp Lobe	GlioMulti 4	15.7	–	100	21.6	45X-Y/46XY	Part exc	+	–	24

No.	Sex	Age	Site	Diagnosis	p53	EGFR	%	Ki-67	Karyotype	Excision			Survival
278	M	37	Right Temp Lobe	Astro 4	0.8 / 0.8 / 6.4	++ / + / +++	100 / 100 / 50 / 50	9.1 / 30.9 / 8.7	Failed	Part exc	+	−	39
108	F	7	Left Front Pariet Lobe	GigGlio	0.0	− / +	100	2.3	Normal	Total exc	+	+	95 alive
18	M	14	Left Front Lobe	Astrobl 3	2.6	+ / ++ / +++	50 / 50	7.5	Failed	Part exc	+	−	143 alive
210	M	23	Right Front	Astrobl 3	0.0	++++	100	0.6	Not Done	Total exc	+	−	51 alive
239	F	62	Left Parasag	Oligo 2	0.0	+ / +++	100	0.1 / 3.7	Failed	Subtot exc	+	−	52 alive
202	M	20	Right Front Lobe	Epend 2	0.0	−	70 / 30	11.6	Failed	Total exc	+	−	52 alive
144	F	21/12	Floor 4th Ventr	Epend 2	0.0	+ / ++	100	2.3	Normal	Total exc	+	+	97 alive
168	M	52	Post Fos / Fil Term	Epend 3	0.0	− / +	100	0.1	Failed	Total exc	−	−	65 alive
229	F	59	Fil Term / Conus L2	EpendMyx	0.0	−	100	0.1	Not Done	Subtot exc	+	−	54 alive
71	F	30	Left Occip Lobe	PNET	26.5 / 0.1	+ / +++	100 / 100	8.9 / 4.8	Not Done	Subtot exc	+	+	113 alive
98	M	14	Cerb Mid	PNET	0.0	−	100	29.7	Normal	Total exc	+	+	105 alive
145	F	5	Right Front / Pariet Lobe	PNET	0.0	+ / +	100 / 80–90	6.7 / 10.2	Normal	Total exc	+	+	93 alive
246	F	27	Cerb Hemi	PNET cystic	0.0	− / +++	10–20 / 75	7.8	Normal	Total exc	+	−	35 alive
288	F	5	Theca L4/5	PNET Metas	0.0	+ / ++	25 / 100	1.1	Normal	Biopsy	+	+	35 alive
289	F	3	Left Front Temp Lobe	PNET	0.0	+ / +++	99 / 1	7.4	Normal	Subtot exc	+	+	32.5
231	M	10/52	Left Lat Ventr	Chor Plex Papill early malignant	0.0	−	100	2.2	Failed	Total exc	−	−	0 other causes
286	M	8/52	Left Chor Plex	Chor Plex Papill malignant	0.0	+	>99 / <1	7.1	45XY-21 or -22	Subtot exc	−	+	31
291	M	55	4th Ventr	Chor Plex Papill non-malignant	0.0	+	90 / 10	1.0	Failed	Total exc	−	−	24 alive
200	M	25	Left Temp Pariet Lobe	Angioglioma 3	0.0	+ / +++	100	0.0	Failed	Part exc	+	−	67 alive
237	M	69	Right Temp Lobe	Angioglioma 4	0.0	++ / +++	100	0.1	45X-Y / 46X-Y + 20	?	−	−	2 other causes
175	M	62	Cerb	Haemangiobl	0.0	++	100	0.7	Not Done	?	+	−	1/7 other causes
221	F	31	Cerb	Haemangiobl	0.0	?		1.2 / 2.8	Normal	Total exc	−	−	56 alive
268	F	38	Medulla	Haemangiobl Recur	0.0	−	100	0.1	Not Done	Part exc	−	−	2/7 other causes
146	F	20	Post Fos ExDur	Dysgerminoma	3.9	− / +	85–90 / 10–15	13.5	Normal	Biopsy	−	−	1
227	F	10	Lat Hypo Thal	Dysgerminoma	0.8	−	100	11.1	Failed	Biopsy	+	−	54 alive
283	M	55	Right BasSk	ChondroSarc	0.0	−	100	0.0	Failed	Total exc	−	−	35 alive
52	M	5	Right CPA	Angioma	0.0	−	100	0.0	Normal	Total exc	+	−	134 alive
68	M	45	Pit	Pit Adenoma	0.0	−	100	0.3	Not Done	Part exc	+	−	118 alive
164	M	52	Pit	Pit Adenoma	0.0	−	100	0.6	Failed	Subtot exc	+	+	75 alive
233	M	54	Pit	Pit Adenoma	0.0	−	100	0.7	Failed	Part exc	+	+	50 alive

(continued overleaf)

Table II Univariate analysis (Cox's regression) for age and Ki-67 LI data

Variable	Coefficient	SE	Relative risk	P-value
Age	0.0378	0.0106	1.038	0.0002
Ki-67 LI	0.077	0.021	1.08	0.0001

Table III Univariate analysis (Log-rank test)

Variable	X^2_1	P-Value
Sex (F *vs* M)	0.07	0.79
p53 (+ VE *vs* − VE)	4.44	0.035
EGFR (+ VE *vs* − VE)	7.34	0.007
Ki-67 LI (< 5% *vs* > 5%)	20.50	< 0.0001
Surgery (total *vs* subtotal *vs* partial *vs* biopsy)	$7.37 = X^2_3$[a]	0.06
Histopathology grade (1,2 *vs* 3,4)	18.30	< 0.0001
Radiotherapy (+ *vs* −)	0.62	0.43
Chemotherapy (+ *vs* −)	0.01	0.94

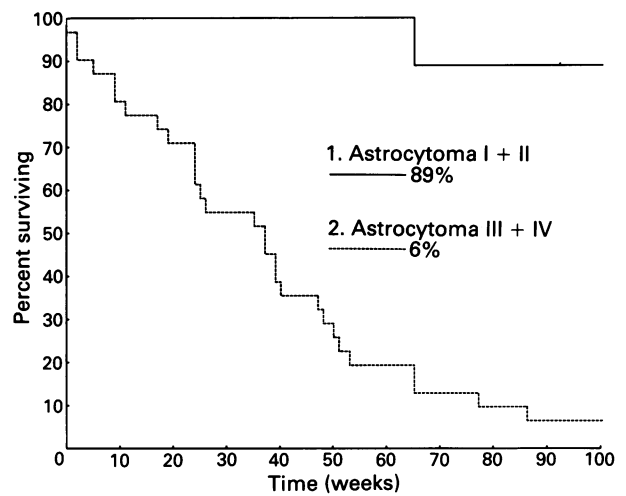[a]Note, 3 degrees of freedom.

Figure 1 Survival curves for patients with astrocytomas grade 1 and 2 (−; $n = 9$) and patients with astrocytoma grade 3 and 4 (---; $n = 31$). Log-Rank statistic = 18.30; $P < 0.0001$; d.f. = 1.

Table I (*continued*)

1 Patient number	2 Sex	3 Age yrs	4 Site of tumour	5 Histological diagnosis and grade (1–4)	6 p53 LI %	7 Immunohistochemical analysis EGFR labelling intensity −to ++++	area %	8 Ki67 LI %	9 Cytogenetic analysis	10 Surgery	11 Radio	12 Chemo	13 Survival time PostOp Weeks
119	F	43	Pit	Metas Carc	1.2	−	100	8.4	Not Done	Part exc	+	−	13
162	F	35	Right Front Lobe	Metas Melanoma	0.9	−	100	6.4	46XX/ Hyperdiploid with markers	Subtot exc	+?	−	21
287	M	31	Right Occip Lobe	Metas Teratoma	0.0	−	100	9.1	Not Done	Subtot exc	+	−	?
91	M	13	Left Front Temp Lobe	NeuroFibroSarc	1.0	− +	99, 1	4.0	Normal	Total exc	+	+	91
269	F	8	Para Vert L?	NeuroFibro	0.0	− +	99, 1	0.2	Normal	Biopsy	−	−	19 alive

Astro = astrocytoma; Astrobl = astroblastoma; Amyg = amygdala; BasSk = base of skull; Cerb = cerebellum; Carc = carcinoma; CATG = categories; Cerb Hemi = cerebellar hemisphere; Cerb Mid = cerebellum midline; ChondroSarc = chondrosarcoma; Chor Plex = choroid plexus; Chor Plex Papill = choroid plexus papilloma; CPA = cerebelo-pontine angle; Epend = ependymoma; EpendMyx = myxopapillary ependymoma; ExDur = extra dural; F = female; Fil Term = filum terminale; Front = frontal; GigGlio = gigantocellular glioma; GlioMulti = glioblastoma multiforme; Haemangiobl = haemangioblastoma; HypoThal = hypothalamus; Int Caps = internal capsule; L2, 4/5 or ? = lumbar segment, 2, 4/5 or ?; Lat = lateral; M = male; Metas = metastatic; NeuroFibro = neurofibroma; NeuroFibroSarc = neurofibrosarcoma; Occip = occipital; Oligo = oligodendroglioma; Pariet = parietal; ParaSag = parasagital; ParaVert = paravertebral; Part exc = partial excision; Pedunc = peduncles; Pit = pituitary; Pit Fos = pituitary fossa; PNET = primitive neuroectodermal tumour; PostOp = post operation; Post = posterior; Post Fos = posterior fossa; Recur = recurrent; Subtot exc = subtotal excision; Temp = temporal; Thal = thalamus; Total exc = total excision; Tuber scl = tuberous sclerosis; Ventr = ventricle.

endothelial proliferation. The nuclear labelling did not correlate with any particular tumour cell type. This was most obvious in such morphologically heterogeneous tumours such as glioblastoma multiforme or gigantocellular glioma where both small and large nuclei were either labelled or unlabelled (Figures 2 and 3). The intensity of the nuclear labelling within the tumour also varied: lesser or more intensely labelled nuclei were intermingled in an irregular fashion (Figures 2 and 3).

Tumour cell nuclei positively labelled with the p53 antibody were found in seven out of 16 types of tumours examined (Table IV, columns 1 and 2). In astrocytomas it should be noted that none of the grade 1 tumours were labelled but that the proportion of positive tumours increased with tumour grade (Table IV, column 3). P53 LI was variable within astrocytoma grades ranging from 1.2% to 29.4% in grade 3, and from 0.1% to 61.9% in grade 4 (Table I, column 6), and the mean LI was similar in the two grades ($P = 0.83$; Table V) indicating absence of correlation between p53 LI and the tumour grade, at least for the two malignant grades. In some tumours a striking regional heterogeneity in p53 + ve nuclei was evident in histological sections. For example, in a p53 + ve PNET (patient number 71) shown in Figure 4, p53 LI in two neighbouring areas was 0.1% and 21.8%. Several astrocytomas grade 3 and 4 also showed regional heterogeneity in the p53 labelling, the greatest differences in p53 LI was 1.2% and 22% in patient number 147 (Table I, column 6).
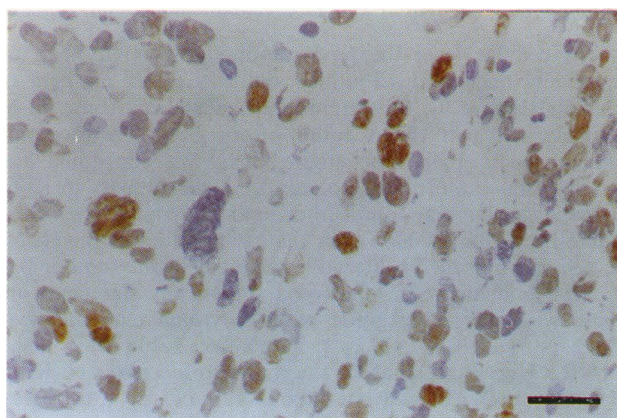
Figure 2 Positive p53 mutant protein nuclear labelling in astrocytoma grade 4 (glioblastoma multiforme) with PAb 1801; both small and large nuclei were either labelled (brown) or unlabelled (blue). Scale bar = 30 μm.
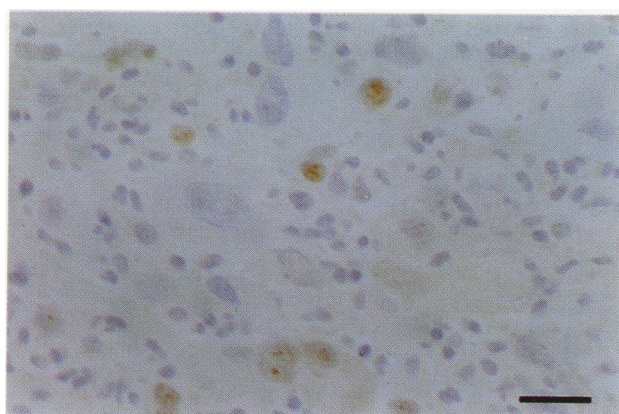

Figure 3 Positive p53 mutant protein nuclear labelling in gigantocellular glioma with PAb 1801; both small and large nuclei were either labelled (brown) or unlabelled (blue). Scale bar = 30 μm.

Table V p53, EGFR, and Ki-67 labelling in relation to histopathological tumour grade

Grade	p53 LI Mean (s.d.;n)	EGFR labelling factor Mean (s.d.;n)	Ki-67 LI Mean (s.d.;n)
1 & 2		110.1(31.6;10) vs 3 & 4: $P<0.0001$ d.f. = 41	1.1(1.0;10) vs 3: $P = 0.01$ d.f. = 21
3	14.1 (12.2;13) vs 4: $P = 0.83$ d.f. = 31	247.6(113.1;13) vs 4: $P = 0.1$ d.f. = 31	8.3(9.1;13) vs4: $P = 0.006$ d.f. = 31
4	12.4(16.3;20)	323.0(136.1;20)	13.4(7.3;20)
3 & 4		293.1(131.2;33)	
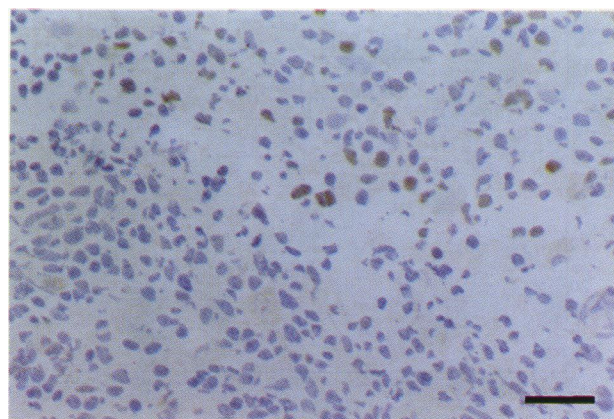
Figure 4 Regional heterogeneity of positive p53 mutant protein nuclear labelling (brown) with PAb 1801 in primitive neuroectodermal tumour: in an area partly shown in the upper half of the figure p53 LI was 21.8%, in a neighbouring area, partly shown in the lower half of the figure, p53 LI was 0.1%. Ki-67 LI quantified from a serial section, was similar in the two areas, about 9%. Scale bar = 30 μm.

Patients with p53 + ve astrocytomas had a reduced survival ($P = 0.035$; Table III) – only 11% of these patients were alive at 100 weeks following operation and diagnosis compared to 36% of patients with p53-ve tumours (Figure 5). The number of cases with tumours in the other categories were too small to attempt survival analyses.
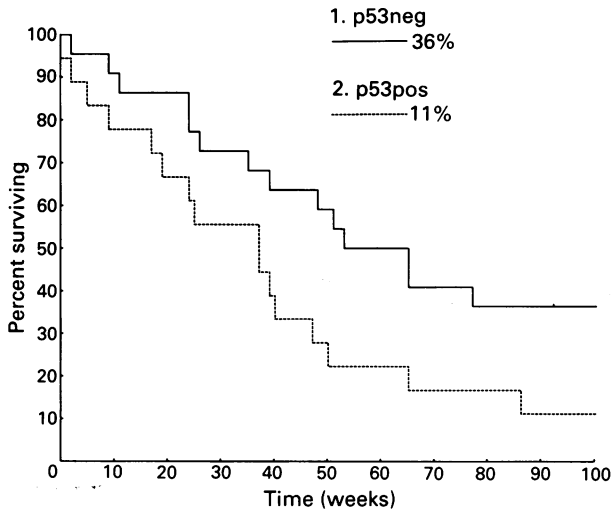
Immunohistochemistry of EGFR

No labelling was seen in normal neocortical (12 ×) and normal cerebellar (4 ×) nervous tissue adjacent to tumours or in tumour endothelial cells. The EGFR labelling of tumours was restricted to cytoplasmic regions and, in some instances, possibly to cell membranes of tumour cells in the EGFR + ve tumours (Figures 6 and 7). This is in contrast to normal human epidermis, which was used to determine the optimal dilution of the EGFR antibody, where the labelling was associated exclusively with cell membranes.

Eleven out of 16 types of tumours examined had EGFR positive cells (Table IV, column 1 and 4). It should be noted that a higher proportion of all astrocytomas was labelled with EGFR (74%) than p53 antibody but that, similar to p53 labelling, none of the grade 1 tumours were labelled, and the proportion of positive tumours increased with tumour grade (Table IV, column 5). In some tumours the EGFR labelling was intense and uniform both in terms of distribution and intensity (Figure 6) but in other tumours it was fainter (Figure 7) or patchy and of variable intensity (see also Table I, column 7). The variability of these two parameters was taken into account by calculating EGFR labelling factor for each tumour (see Material and methods). Astrocytomas grades 3 & 4 had a significantly higher mean labelling factor than grades 1 and 2 ($P<0.0001$; Table V) indicating that the intensity/area of EGFR labelling increased with malignancy

Table IV Expression of p53 and EGF receptor proteins in CNS tumours

1 Tumour type	2 p53 + ve Ratio	3 %	4 EGFR + ve Ratio	5 %
Astrocytoma Grade 1	0/4	0%	0/4	0%
Grade 2	1/6	17%	2/6	33%
Grade 3	5/13	38%	11/13	85%
Grade 4[a]	13/20	65%	9/20	95%
Total	19/43	44%	32/43	74%
Primitive neuro-ectodermal tumours	1/6	17%	4/6	67%
Astroblastoma	1/2		2/2	
Gigantocellular glioma	1/1		1/1	
Oligodendroglioma	0/1		1/1	
Ependymoma Grade 2/3	0/2		2/2	
Myxopapillary	0/2		0/2	
Angioglioma	0/3		1/2	
Choroid plexus papilloma	0/3		3/3	
CNS dysgerminoma	2/2		1/2	
Pituitary tumour	0/3		0/3	
Haemangioblastoma	0/3		2/3	
Chordoma	0/1		0/1	
Tumours in Von Recklinghausen's				
neurofibroma[b]	0/1		1/1	
neurofibrosarcoma[c]	1/1		1/1	
Angioma (developmental abnormality)	0/1		0/1	
Metastatic tumours in CNS				
carcinoma	0/2		0/1	
melanoma	1/1		0/1	

[a]Includes glioblastoma multiforme. [b]Associated with spinal roots. [c]Intrinsic to cerebrum.

grade. Other tumours with high degree of EGFR labelling included PNETs, astroblastomas, oligodendrogliomas, choroid plexus papillomas, and angiogliomas (Table I, column 7).

Patients with EGFR + ve astrocytomas appeared to have reduced survival ($P = 0.007$; Table III) – only 13% of these patients were alive at 100 weeks following diagnosis compared to 60% of EGFR-ve patients (Figure 8). The number of cases with tumours in the other categories were too small to attempt survival analysis.

Immunohistochemistry of Ki-67

At the cellular level the Ki-67 antibody reactivity had an exclusively nuclear distribution, and was either uniform or granular (Figure 9). Most tumours had at least some Ki-67 labelled nuclei with the exception of one grade 2 astrocytoma in tuberous sclerosis, one angioglioma, one chordoma and and one angioma (Table I, column 8). Heterogeneity in Ki-67 nuclear labelling was found in around of 20% astrocytomas (Table I, column 8). However, the mean values of the Ki-67, LI, representing the proliferative potential of the tumour (see Material and methods), showed a statistically significant increase with increasing grade of astrocytoma malignancy (1 and 2 *vs* 3; $P = 0.01$; 3 *vs* 4: $P = 0.006$; Table V).

In the Univariate analysis the astrocytoma patients' Ki-67 LI, when analysed as a continuous variable by Cox regression analysis, showed a strong relationship to the length of surival ($P > 0.0001$; Table II). When analysed by the Log-Rank test patients with Ki-67 LI $> 5\%$ (5.1 to 30.9%) had a reduced survival ($P < 0.0001$; Table III) – none of these patients survived beyond 86 weeks following diagnosis compared with 63% of patients with Ki-67 LI of $< 5\%$ (0.1 to 3.9%) who were still alive at 100 weeks (Figure 10). The number of cases with tumours in the other categories were too small to attempt survival analysis.
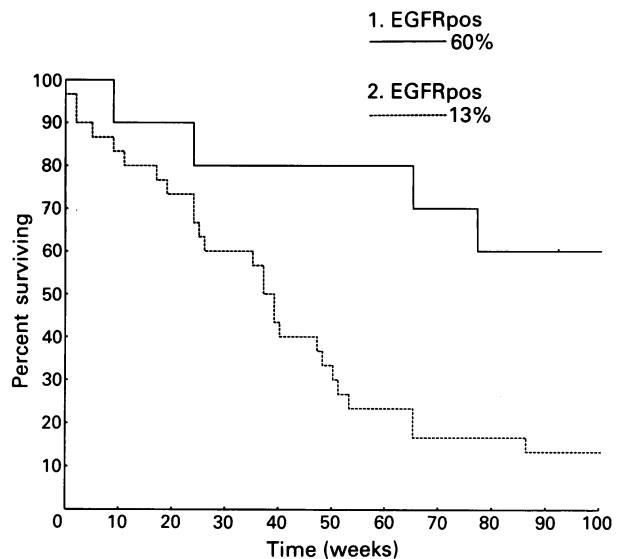

Figure 5 Survival curves for patients with p53 negative astrocytomas (p53neg–; $n = 22$) and patients with p53 positive astro cytomas (p53pos---; $n = 18$). Log-Rank statistic = 4.44; $P = 0.035$; d.f. = 1.

Figure 6 Intense positive EGFR labelling (brown) in astrocytoma grade 4 (glioblastoma multiforme) with EGFR1 antibody; the blood vessel (v) is unlabelled. Scale bar = 30 μm.

Figure 8 Survival curves for patients with EGF receptor negative astrocytomas (EGFRneg–; $n = 10$) and patients with EGF receptor positive astrocytomas (EGFRpos---; $n = 30$). Log-Rank statistic = 7.34; $P = 0.007$; d.f. = 1.
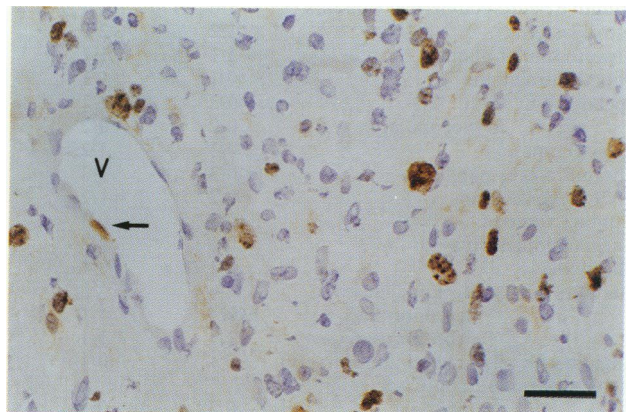

Figure 7 Faint EGFR labelling (pale brown) in gigantocellular glioma with EGFR1 antibody. Scale bar = 30 μm.

Figure 9 Positive Ki-67 nuclear labelling (brown) in astrocytoma grade 4 (glioblastoma multiforme); a blood vessel (v) displays one labelled endothelial cell nucleus (arrow). Scale bar = 30 μm.
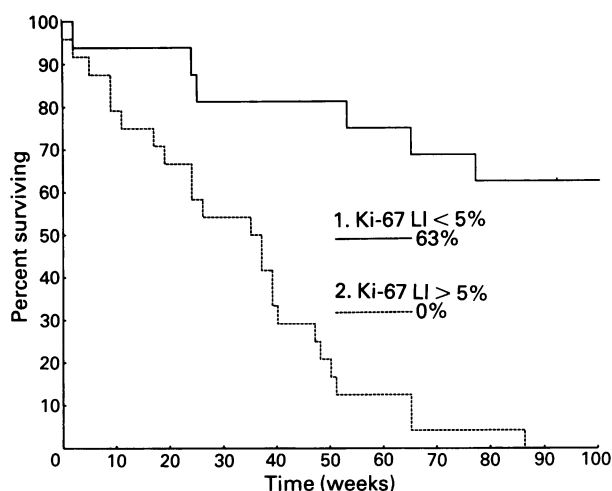
Figure 10 Survival curves for patients with Ki-67 LI below 5% ($<5\%-$; $n = 16$) and patients with Ki-67 LI above 5% ($>5\%$ ---; $n = 24$). Log-Rank statistic = 20.50; $P<0.0001$; d.f. = 1.

Relationship between p53, EGFR and Ki-67 labelling

In about 25% of the astrocytomas regional heterogeneity was detected either in p53, EGFR, or Ki-67 labelling, and therefore more than one area was counted in those tumours (Table I, columns 6, 7, 8). Note that in most cases only one of the three antibodies showed heterogeneity, in a few cases two antibodies but never all three simultaneously. The relationship between p53, EGFR and Ki-67 labelling was determined in serial sections from corresponding areas but not between different areas. The p53 + ve astrocytomas had a significantly higher mean Ki-67 LI than p53-ve astrocytoma ($P = 0.036$; Table VI), although the values of the continuous variables, p53 LI and Ki-67 LI, did not show any correlation within the individual tumours ($r = 0.19$; $P = 0.17$). A pictorial example of absence of correlation should be noted in Figure 4 where regional heterogeneity in the p53 labelling cannot be explained by differences in proliferative activity between the two areas since they both had an almost identical Ki-67 LI of 9%.

The continuous variables, EGFR labelling factor and Ki-67 LI, were significantly correlated within the individual tumours ($r = 0.32$; $P = 0.018$). The EGFR + ve astrocytomas had a significantly higher mean Ki-67 LI than EGFR-ve astrocytomas ($P = 0.028$; Table VI). Astrocytomas which were both EGFR + ve and p53 + ve had a somewhat higher Ki-67 LI than astrocytomas which were EGFR + ve but p53-ve, though the difference was not significant ($P = 0.49$; Table VI). Astrocytomas not expressing either EGFR or p53 proteins had the lowest mean Ki-67 LI ($P<0.0001$; Table

VI), although the continuous variables, p53 LI and EGFR labelling factor, showed no correlation within individual tumours ($r = 0.04$; $P = 0.86$). The absence of correlation is probably due to variability in p53 LI between tumours (see Table I, column 6), and also due to non-overlapping regional heterogeneity in EGFR and p53 labelling within individual tumours.

The effect of EGFR and p53 expression on patient's survival was not cumulative ($P = 0.66$; Figure 11) – similar proportions of patients with EGFR + ve & p53 + ve tumours (12%) were alive at 100 weeks following diagnosis compared to patients with EGFR + ve & p53-ve tumours (15%). In contrast, patients with EGFR-ve & p53-ve tumours had a significantly better survival rate than both of the previous groups (67%; $P = 0.016$; Figure 11).

Multivariate analysis

The list of all variables which were analysed for prognostic importance by univariate analysis are shown in Table II (Cox's Regression for continuous variables) and in Table III (Log-Rank test for categorised variables). Note that the univariate prognostic importance of Ki-67 LI is of the same order of magnitude ($P<0.0001$) whether it is considered as a
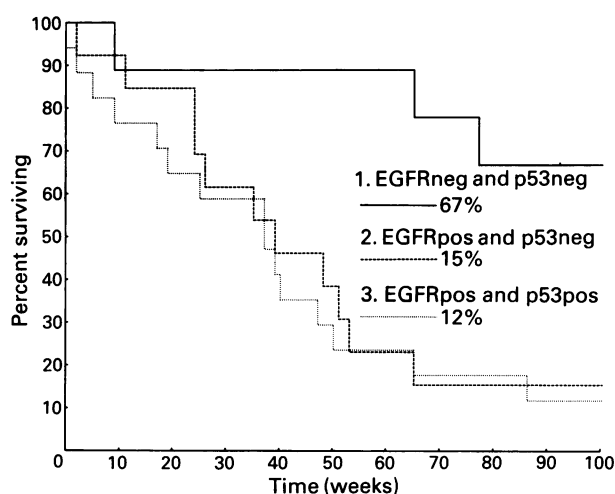

Figure 11 Survival curves for patients with p53 negative and EGFR negative astrocytomas (EGFRneg and p53pos-; $n = 9$), patients with p53 negative and EGFR positive astrocytomas (EGFRpos and p53neg ---; $n = 13$), and patients with p53 positive and EGFR positive astrocytomas (EGFRpos and p53pos . . .; $n = 17$). Log-Rank statistic = 8.29; $P = 0.016$; d.f. = 2. The only patient with a p53 positive and EGFR negative astrocytoma died 24 weeks after diagnosis. For EGFRpos and p53neg vs EGFRpos & p53pos survival curves the Log-Rank statistic = 0.19; $P = 0.66$; d.f. = 1.

Table VI Ki-67 labelling in relation to p53 and EGFR labelling

	Ki-67 LI Mean (SD;n)	P-value	Degrees of freedom
p53-ve	6.2(6.6;24)		
	vs	0.036	41
p53 + ve	11.8(10.2;19)		
EGFR-ve	4.1(6.3;11)		
	vs	0.028	41
EGFR + ve	10.7(8.8;32)		
p53-ve & EGFR + ve	9.0(7.3;14)		
	vs	0.49	30
p53 + ve & EGFR + ve	11.2(10.2;18)		
p53-ve & EGFR-ve	2.3(2.7;10)		
	vs	<0.0001	40
EGFR + ve (= p53-ve & EGFR + ve plus p53 + ve & EGFR + ve)	10.7(8.8;32)		
p53 + ve & EGFR-ve	21.6 (1)		

continuous or categorical ($<5\%$ *vs* $>5\%$) variable. Sex, radiotherapy and chemotherapy had non-significant univariate *P*-values, while surgery was of weak statistical importance ($P = 0.06$; Table III). All variables apart from sex, radiotherapy and chemotherapy were entered into a multivariate analysis to determine whether they influence the patient's prognosis independently or are associated with each other. The multivariate analysis was performed by using a forward stepwise application of Cox's Regression model. Tables VII and VIII shows that the only variables selected as statistically significant ($P<0.10$) by this procedure were histopathological grade and Ki-67 LI. Although several of the variables had a significant prognostic importance following univariate analysis (Tables II and III), the multivariate analysis reveals histopathological grade as the overwhelming dominating factor with Ki-67 LI being the only other variable with prognostic information once histopathological grade has entered the model. By employing the regression procedure with only Ki-67, EGFR and p53 labelling as independent variables, only Ki-67 labelling was significant ($P = 0.0002$), and therefore the controlling variable. This is because many of the variables do not influence the survival of astrocytoma patients independently, and are interrelated with each other as follows, histopathological grade and age: 1 and 2 *vs* 3 and 4; $P<0.0001$; histopathological grade and Ki-67 LI: 1 and 2 *vs* 3: $P = 0.01$, 3 *vs* 4; $P = 0.006$; histopathological grade and EGFR labelling factor: 1 and 2 *vs* 3 and 4: $P<0.0001$; age and Ki-67 LI: $r = 0.37$, $P = 0.014$; age and EGFR labelling factor: $r = 0.57$, $P<0.0001$; Ki-67 LI and EGFR labelling factor: $r = 0.32$, $P = 0.018$; except for histopathological grade and p53 LI: $P = 0.83$; age and p53 LI: $r = 0.06$, $P = 0.77$; Ki-67 LI and p53 LI: $r = 0.19$, $P = 0.17$; and EGFR labelling factor and p53 LI: $r = 0.04$, $P = 0.86$.

Discussion

This study demonstrates immunohistochemically detectable levels of p53 protein in tumour cell nuclei of many CNS and non-CNS tumours. The PAb 1801 monoclonal antibody used in this study can recognize both normal and mutant forms of human p53 proteins (Banks *et al.*, 1986; Rodrigues *et al.*, 1990) but the labelling almost certainly represents accumulation of nonfunctional p53 mutants only. The mutants are detectable by immunohistochemistry (Cattoretti *et al.*, 1988; Iggo *et al.*, 1990; Rodrigues *et al.*, 1990) because of their metabolic stability, and their cellular levels are elevated

10–100 fold above normal values (Finlay *et al.*, 1989). In the present series none of the normal CNS tissue adjacent to the tumours was labelled with PAb 1801 antibody. Also, the labelling of tumour cells is unlikely to represent the somewhat elevated levels of normal p53 seen in actively proliferating cell populations (Dippold *et al.*, 1981; Levin & Momand, 1990) because some tumours with large growth fractions were not labelled with the PAb 1801 antibody, whilst in p53 + ve tumours the p53 LI did not correlate with the growth fraction size. In addition, no endothelial cells were labelled in any of the tumours, even in areas where endothelial proliferation, and Ki-67 labelling were present.

Our finding that p53 mutants are expressed in astrocytomas, primitive neuroectodermal tumour, astroblastomas, gigantocellular glioma, neurofibrosarcoma, CNS dysgerminomas, and melanoma extends the number of human tumours with identified mutations in p53 gene. So far, the list included carcinomas of the breast, lung, colorectum, and liver, and also neurofibrosarcoma, osteosarcoma and glioblastomas multiforme (Masuda *et al.*, 1987; Cattoretti *et al.*, 1988; Nigro *et al.*, 1989; Iggo *et al.*, 1990; Rodrigues *et al.*, 1990; Menon *et al.*, 1990; Bressac *et al.*, 1991; Hsu *et al.*, 1991). Using karyotyping we have not detected abnormalities of chromosome 17p (where gene p53 is localised) in any of the p53 + ve tumours in this study. This may not be surprising since accumulation of p53 protein is almost certainly an outcome of point mutations in the p53 gene (Nigro *et al.*, 1989) which is beyond resolution of karyotyping.

In the present series the p53 labelling was exclusively localised in tumour cell nuclei, whilst in other tumour types either nuclear or a combined nuclear and cytoplasmic p53 labelling has been found (Iggo *et al.*, 1990; Rodrigues *et al.*, 1990). The nuclear localisation may indicate presence of transforming p53 mutants. Normal p53 protein is thought to have a role in regulating gene expression or DNA replication (Michalovitz *et al.*, 1991), and it appears that nuclear localisation of p53 mutants is essential for their transforming activity (Shaulsky *et al.*, 1990). The reason for some p53 mutants accumulating in the nuclei is unclear. Cytoplasmic accumulation of some p53 mutants has been reported to occur because a conformational change in their molecule leads them to form complexes with cytoplasmic heat-shock-cognate protein 70 (hsc 70; Sturtzbecher *et al.*, 1988). It would be of interest to see whether the p53 mutants that accumulate in nuclei of different tumour types in this and other studies share a particular conformational change, and bind to an as yet unidentified nuclear protein.

In astrocytomas this study has found that the nuclear

Table VII Variables that achieved $P<0.10$ following forward stepwise Cox regression on all variables, Ki-67 LI analysed as continuous variable

Variable	Coefficient	SE	Relative risk (95% C.I.)[a]	P-value	χ^2_1 to enter model
Histological grade	2.920	1.050	18.50 (2.37,145.2)	0.005	26.23
Ki-67 LI	0.044	0.025	1.045 (0.99,1.10)	0.08	2.99

[a]95% C.I. = 95% Confidence Interval for Relative Risk is given by $e^{\text{coefficient} \pm 1.96SE(\text{coefficient})}$.

Table VIII Variables that achieved $P<0.10$ following forward stepwise Cox regression on all variables, Ki-67 LI categorical $<5\%$ *vs*$>5\%$

Variable	Coefficient	SE	Relative risk (95% C.I.)[a]	P-value	χ^2_1 to enter model
Histological grade	2.694	1.082	14.80 (1.78,123.2)	0.012	26.23
Ki-67 LI	0.975	0.508	1.921 (0.98,7.17)	0.055	4.31

[a]95% C.I. = 95% Confidence Interval for Relative Risk is given by $e^{\text{coefficient} \pm 1.96SE(\text{coefficient})}$.

expression of p53 mutants was associated with increase in tumour malignancy and poor prognosis. Using immunohistochemistry, similar observations, though without survival data, have been made in neurofibrosarcoma, and carcinomas of the breast, lung, and colorectum (Baker et al., 1989; Vogelstein et al., 1989; Iggo et al., 1990; Harris et al., 1990; Menon et al., 1990). In astrocytomas p53 expression has not yet been examined by immunohistochemistry but allele loss of chromosome 17p, and presumed mutations in p53 gene, have been reported to be associated with tumour initiation (James et al., 1989; El-Azouzi et al., 1989) which contrasts with the findings of the present study. The reason for this apparent difference between conclusions of the present and previous studies may be due to the difference in sensitivities of the different techniques employed, and may not emerge until the mechanism of tumour evolution in astrocytomas is more precisely understood at molecular level. However, several possible explanations might be considered. Firstly, low and high grade astrocytomas may originate from different tumour precursor cells (James et al., 1988). The precursor cells giving rise to low grade astrocytomas may be affected by loss-of-function p53 mutations leading to a failure to express any p53 RNA and protein, similar to that reported in a proportion of rhabdomyosarcomas, osteosarcomas, and Li-Fraumeni lesions (Masuda et al., 1987; Mulligan et al., 1990; Malkin et al., 1990). Only precursors giving rise to high grade astrocytomas may be affected by transforming p53 mutations leading to overexpression of the p53 mutants, similar to that reported in carcinoma of the breast and lung (Cattoretti et al., 1988; Iggo et al., 1990). This scheme, however, implies that benign astrocytomas cannot progress to a malignant stage, which is contrary to clinical and histopathological observations (Russell & Rubinstein, 1989). Alternatively, if astrocytomas progress from low to higher grades (James et al., 1988), they may do so by step-wise changes in the p53 gene, analogous to those proposed for colorectal carcinomas: the first or initiating step involving mutation in one allele only and a synthesis of inactive mutant/normal oligomers; further loss of control is believed to result from deletion of the normal allele, leaving the cell with only a mutant allele (Nigro et al., 1989). The present immunohistochemical essay has detected p53 mutant molecules, but may not have been sufficiently sensitive to detect cells expressing oligomeric p53 mutant/normal molecules, similar to observation made by Rodrigues et al. (1990) on cell lines derived from acute lymphoblastic leukaemia. The finding that p53 LI was variably expressed between tumours, and regionally heterogeneous within tumours, indicates that p53 mutant molecules were expressed in subclones of astrocytic tumour cells, which were not present at the initial stages of tumour development. If a precursor cell expressed p53 mutants, all the daughter tumour cells in high grade astrocytomas would also express the mutant molecules.

In this series EGFR was expressed in astrocytomas and ten other tumour types, mostly with glial and/or neuroepithelial differentiation, but not in normal brain tissue adjacent to the tumours. In astrocytomas EGFR expression was associated with increase in tumour malignancy and poor prognosis. Our data confirm findings of previous biochemical (Liberman et al., 1984; 1985) and immunohistochemical studies on astrocytomas (Reifenberger et al., 1989), and extend them by survival data. But, unlike human breast cancer (Harris et al., 1990), this series does not support a direct association between expression of EGFR and p53 mutants: there was no correlation between p53 LI and categories of EGFR labelling, possibly due to nonoverlapping regional heterogeneity in each parameter. This suggests that in astrocytomas p53 mutants and EGFR are expressed in different subclones of tumour cells, and that the associations between increase in tumour malignancy and the expression of EGFR protein or p53 mutants occur independently of each other.

Overexpression of EGFR, previously found to occur in a high proportion of malignant gliomas, has been related either to an amplification of the EGFR gene, often in the form of double minutes (Liberman et al., 1984; 1985; Wong et al.,

1987), or to an extra copy of chromosome 7 (Liberman et al., 1984) on which the EGFR gene is located (Shimizu et al., 1985), or to loss of control of transcriptional activity of the gene (Gerosa et al., 1989). In this study trisomy of chromosome 7 was found only in one patient, and double minutes in none. It therefore seems that in the present series the possible mechanisms responsible for EGFR overexpression are either amplification at the EGFR gene locus which is not easily detectable by karyotyping, or loss of control of transcriptional activity. Our observation that in the majority of the brain tumours EGFR labelling had a predominantly cytoplasmic distribution may be explained by rapid internalisation of the EGR after ligand binding (Stoscheck & King, 1986; Humphrey et al., 1990). Alternatively, similar to human glioma cell lines which show co-expression of high levels of EGFR and one of its ligands TGF-α (Nister et al., 1988), an autocrine growth stimulation loop may operate in astrocytomas in vivo, and the cytoplasmic labelling may represent cytoplasmic binding of EGFR to its ligand.

In this series high Ki-67 LI was associated with reduced survival. This is contrary to the only previous study which included survival data (Zuber et al., 1988), possibly due to a small sample size in the previous study. It is likely that the poor prognosis found in patients with Ki-67 LI higher than 5% reflects a significant correlation between mean size of the growth fraction, as determined with the Ki-67 index, and histopathological grade of malignancy in astrocytomas. The latter observation is in broad agreement with previous studies (Raghavan et al., 1990; Brown & Gatter, 1990) but the conclusions of the present study go further – the Multivariate analysis has demonstrated that the histopathological grade is the most important variable to influence the patient's survival, when all variables are considered together. The analysis has also demonstrated that histopathological grade, age, Ki-67 LI, and EGFR and p53 labelling do not influence survival of astrocytoma patients independently but, except for the p53 labelling, are interrelated with each other. The proliferative ability of astrocytoma cells, as determined by Ki-67 LI, appeared to be positively influenced by expression of both p53 mutants and EGFR protein, since p53 + ve or EGFR + ve astrocytomas had significantly higher Ki-67 indices than p53-ve or EGFR-ve astrocytomas but the Ki-67 LI is the most important variable to influence the patient's survival when considered together with the EGFR and p53 labelling. This finding and two other observations indicate that additional or alternative mechanisms to expression of p53 mutants and EGFR are likely to be involved in controlling tumour cell proliferation and tumour progression in astrocytomas. Firstly, in p53-ve and/or EGFR-ve astrocytomas tumour cells also displayed proliferative activity, even though it was lower than in p53 + ve and/or EGFR + ve tumours. Secondly, though EGFR labelling correlated positively with Ki-67 LI, analysis of p53 LI and Ki-67 LI did not show a significant correlation. These conclusions are in keeping with earlier cytogenetic findings which have implicated multiple chromosomal abnormalities in malignant progression of gliomas in addition to 17p and 7, where p53 and EGFR genes respectively, are localised (Bigner et al., 1984; Shapiro, 1986; James et al., 1988). In model for progression of gliomas Bigner and Vogelstein (1990) proposed that abnormality on chromosome 17p occurs at early stages of tumorigenesis, whilst EGFR is thought to stimulate further progression of malignant gliomas. This study has demonstrated that in astrocytomas expression of both p53 mutants and EGFR can occur at early stages of tumorigenesis, and that their expression also represents mechanisms associated with malignant progression and poor prognosis but that expression of neither protein may be essential for this process.

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