PAPER

Molecular-genetic characterisation of gliomas that recur as same grade or higher grade tumours

T J M Hulsebos, D Troost, S Leenstra

J Neurol Neurosurg Psychiatry 2004;**75**:723–726. doi: 10.1136/jnnp.2003.025031

See end of article for authors' affiliations

Correspondence to: Dr T J M Hulsebos, Institute of Human Genetics, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, the Netherlands; t.j.hulsebos@amc.uva.nl

Received 31 July 2003
In revised form 5 October 2003
Accepted 7 November 2003

Background: Due to their invasive growth, gliomas usually cannot be removed completely and almost always recur as same grade or higher grade malignancies.
Objective: To determine whether there were differences in the accumulation of genetic changes between the two types of glioma recurrence.
Methods: We genetically characterised 14 cases of lower grade glioma with a same grade recurrence, 12 cases of glioblastoma recurrence, and 14 cases of lower grade glioma with a higher grade recurrence. We investigated LOH (loss of heterozygosity) at 1p36, 10p15, the *PTEN* region in 10q23, the *DMBT1* region in 10q25, 19q13, 22q13, LOH and mutation of *TP53*, and *EGFR* amplification.
Results: Genetic heterogeneity in the primary tumour was inferred in 3 cases of lower grade glioma with a higher grade recurrence. The cases of lower grade glioma with a higher grade recurrence displayed increased genetic instability in the recurrence (mean of 2.0 additional genetic changes per case) compared to cases with a same lower grade recurrence or those with a glioblastoma recurrence (mean of 0.6 and 0.8 additional changes per case, respectively). Compared to unselected primary glioblastomas, the glioblastomas that recurred as an operable tumour had infrequent *EGFR* amplification (8% *v* 30–40% of cases).
Conclusions: Gliomas recurring as higher grade lesions might be genetically heterogeneous and accumulate more genetic changes than gliomas recurring as same grade lesions (whether originally low or high grade). Primary glioblastomas from patients for which the recurrence is operated because of prognostically more favourable clinical indices have infrequent *EGFR* amplification.

INTRODUCTION

Gliomas are the most common primary neoplasms of the central nervous system.[1] Their capacity to invade surrounding normal brain prevents complete removal of the tumour. Despite intensive radio- or chemotherapy, there is almost always recurrence of the tumour. Gliomas usually recur as a same grade or as a higher grade lesion. However, the time to recurrence and the type of the recurrent tumour are highly variable and, in general, cannot be predicted from the histopathological and clinical characteristics of the primary tumour.

Glioma tumourigenesis is thought to be the consequence of the accumulation of genetic changes that confer growth advantage to a glial cell. Important genetic changes in gliomas are loss of heterozygosity (LOH) at 1p, 9p, 10p, 10q, 13q (with *RB1*), 17p, 19q, and 22q, *EGFR* amplification, homozygous deletion of *CDKN2A/B* on 9p and *DMBT1* on 10q, and mutation of *PTEN* on 10q and *TP53* on 17p.[2] Earlier, we studied genetic changes associated with glioma recurrence in a limited series of cases with same grade or higher grade recurrences.[3] We concluded that glioma recurrence is characterised by the increased involvement of tumour suppressor genes or gene regions, even in those cases in which the primary and the recurrent tumour were of the same (high) malignancy grade. These series did not allow us to draw conclusions about possible preferences with regard to the type of genetic changes involved in glioma recurrence or possible differences in accumulation rate of these genetic changes between the two types of recurrence. To investigate this, we extended our analyses by the inclusion of a substantial number of new cases of both types of recurrence. We specifically studied LOH at 1p36, 19q13, 10p15, 10q23 with *PTEN*, and 10q25 with *DMBT1*, LOH and mutation of *TP53*, and *EGFR* amplification as indicators of genetic instability because these are well known genetic changes that occur rather frequently in gliomas. In addition, we analysed LOH at 22q13 because we, and others, have recently given evidence for the presence of a tumour suppressor gene in that region that might be involved in astrocytoma progression.[4 5]

METHODS

Tumour samples and clinical data

Tumour samples were obtained from 40 pairs of primary gliomas and one or more recurrences. Of these, 18 have been analysed for genetic changes previously.[3] All tumour samples were classified and graded according to the World Health Organization (WHO) criteria.[1] DNA was extracted from frozen samples or from formalin fixed, paraffin embedded sections according to standard methods. The clinical data for the patients are given in table 1.

LOH analysis

Primer sequences for amplification of markers and genes and conditions for polymerase chain reaction (PCR) were taken from the Genome Database (www.gdb.org/). The LOH status of the chromosomal regions was determined by analysing the markers that we used in our previous study[3] and the following additional markers: D1S468/1795, D19S1180/1182, *PLA2G4C*, D10S186/212/215/249/541/542/587/597/608/1234/1716/1745/, *PTENCA*, D22S270/274/282/418/922/928/1141/1149/1151/1169/1170. The LOH status of each region was inferred from the data of at least two informative markers.

Abbreviation: LOH, loss of heterozygosity

Table 1 Genetic changes in cases with same lower grade, glioblastoma, and higher grade recurrences

Case*	Age†/ Sex	Histology‡ P/R/RR	Treat-ment§	Interval¶ (months)	Loss of heterozygosity (LOH)†† 1p36	19q13	10p15	*PTEN*	*DMBT1*	LOH *TP53*	MUT** *TP53*	LOH 22q13	*EGFR* Ampl
Same lower-grade recurrences													
2020	27/M	DA/DA	–	17	n	n	n	n	n	n	n	n	n
2228	52/M	DA/DA	–	29	n	n	n	n	y	y	n	n	n
2509	39/F	DA/DA	RT	143	y	y	n	n	n	y	**n/y**	n	n
1112	22/F	OA/OA	–	14	y	y	n	n	n	n	n	n	n
305 (8)	38/M	AO/AO/AO	RT/–	26/15	y	y	n	n	n	n	n	n	n
347 (9)	28/F	AO/AO	–	53	y	y	n	n	n	n	n	y	n
814	62/M	AO/AO	RT	27	y	y	y	n	n	y	n	y	n
2250	47/M	AO/AO	–	9	y	y	n	n	n	n	n	n	n
2729	35/M	AO/AO	RT, CT	6	n	y	y	y	n	n	n	**n/y**	**n/y**
831 (10)	26/M	AA/AA	RT	36	n	n	**n/y**	n	n	y	**n/y**	**n/y**	n
1113	52/V	AA/AO	RT	92	n	y	n	y	y	y	y	n	n
1568	27/M	AA/(A)OA	RT	56	y	y	n	n	n	n	n	n	n
2404 (11)	31/F	AA/AA/AOA	RT/CT	5/53	y	y	**n/n/y**	**n/n/y**	**n/n/y**	y	y	n	n
2848	29/M	AOA/AO	CT	14	y	y	n	n	y	y	n	n	n
Glioblastoma recurrences													
663 (13)	36/F	GBM/GBM		6	n	y	n	n	n	y	y	n	n
871 (19)	51/F	GBM-glios/GBM-glios	RT,BR	11	n	n	y	y	**n/y**	n	y	n	n
1143 (15)	42/M	GBM/GBM	RT/BR	9	n	n	n	n	n	n	n	n	y
1959 (12)	39/F	GBM/GBM	RT	45	n	n	n	n	n	n	n	n	n
1857 (14)	35/M	GBM/GBM	RT	40	n	n	n	n	n	n	y	n	n
1892 (17)	63/M	GBM/GBM	RT	8	**n/y**	n	n	**n/y**	**n/y**	n	n	n	n
2176 (18)	60/F	GBM/GBM	RT,BR	22	n	n	n	n	n	n	n	n	n
2403	26/M	GBM/GBM	RT	20	n	n	n	n	n	n	n	n	n
2406	45/F	GBM-O/GBM	RT	36	y	**n/y**	y	y	y	n	y	**n/y**	n
2516	41/M	GBM-glios/GBM-glios	BR	11	n	**n/y**	y	y	y	y	y	n	n
2656	57/M	GBM/GBM/GBM	RT, CT/–	3/22	n	**n/n/y**	y	y	y	**n/n/y**	n	n	n
2884	55/M	GBM/GBM-O	RT	6	**n/y**	y	y	y	y	**n/y**	n	y	n
Higher grade recurrences													
2308 (1)	35/F	PA/GBM	–	12	n	**n/y**	n	**n/y**	**n/y**	**n/y**	y	**n/y**	n
2696	17/M	DA/DA/AA	–/–	62/27	n	n	**n/n/y**	**n/n/y**	**n/n/y**	**n/n/y**	y	**n/n/y**	n
1415 (2)	26/M	DA/AA	–	16	n	y	n	n	y	n	y	n	n
1454	29/M	DA/AA	–	88	n	n	n	n	n	y	y	n	n
1748 (3)	21/M	DA/AA	–	11	n	n	n	n	n	y	y	n	n
2540	40/F	DA/AA	–	37	n	n	n	n	n	y	y	n	n
2537	43/M	DA/AOA	–	33	**n/y**	**n/y**	n	n	**n/y**	n	y	n	n
1261 (5)	23/F	DA/GBM	–	58	n	n	n	n	n	y	y	n	n
912 (4)	50/M	DA/GBM-PNET	–	12	n	**n/y**	n	n	n	**n/y**	y	**n/y**	n
1083	45/F	O/AO	RT	25	**n/y**	n	**imb/y‡‡**	n	**n/y**	y	y	**imb/y‡‡**	n
2778	26/M	OA/O/AO	–/RT	11/40	y	y	n	n	n	n	y/n/n	n	n
1140 (6)	31/M	AA/AA/AAless diff	RT/–	37/14	n	**n/n/y**	**n/y/y**	n	**n/n/y**	n	y	n	n
2111	21/F	AA/GBM	RT	56	n	n	**y /y‡‡**	n	n	n	**n/y**	n	n
1866 (7)	36/M	AA/GBM/GBM	RT/–	8/9	n	n	**n/n/y**	**n/n/y**	**n/n/y**	y	y	n	n

*Number in parentheses denotes case number in a previous communication[3]
†Age (years) at first operation
‡P, primary tumour; R, first recurrence; RR, second recurrence; PA, pilocytic astrocytoma; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; GBM-PNET, primitive neuroectodermal tumour-like glioblastoma; GBM-glios, mixed glioblastoma-gliosarcoma; GBM-O, glioblastoma with oligodendroglial features; (A)O, (anaplastic) oligodendroglioma; (A)OA, (anaplastic) oligoastrocytoma
§RT, radiotherapy; BR, brachytherapy; CT, chemotherapy
¶Interval between operations
** Type and position of *TP53* mutation were reported previously[3] or can be obtained from the corresponding author (T.H.)
††A y denotes presence and n absence of the genetic change. Cases with additional genetic changes are shown in bold.
‡‡See table 2 for details. Imb denotes imbalance.

Table 2 Marker allele diminished or lost in primary tumour (P) or recurrence (R)

Case	P/R	Marker	
1083		(10p15)	
		D10S249	D10S559
	P	bottom dim	top dim
	R	top lost	bottom lost
1083		(22q13)	
		D22S1151	D22S282
	P	bottom dim	top dim
	R	top lost	bottom lost
2111		(10p15)	
		D10S1234	D10S1435
	P	top lost	top lost
	R	bottom lost	bottom lost

TP53 mutation and *EGFR* amplification

Exons 5 and 6 of *TP53* were directly sequenced. Exons 7 and 8 were first screened for the presence of mutations by denaturing gradient gel electrophoresis,[6] which was followed by direct sequencing of the mutation-containing exon. Amplification level of *EGFR* was determined as described previously.[7]

RESULTS

Forty pairs of primary tumour and one or more recurrent tumours were analysed for LOH at 1p36, 19q13, 10p15, the *PTEN* region in 10q23, the *DMBT1* region in 10q25, LOH and mutation of *TP53*, LOH 22q13, and *EGFR* amplification. These genetic changes were studied because they frequently occur in gliomas and because they have prognostic and diagnostic significance for certain types of glioma.[4 5 8–10] Except for LOH 22q13, which was newly determined for all cases in this study, the genetic changes for 18 cases were taken from a

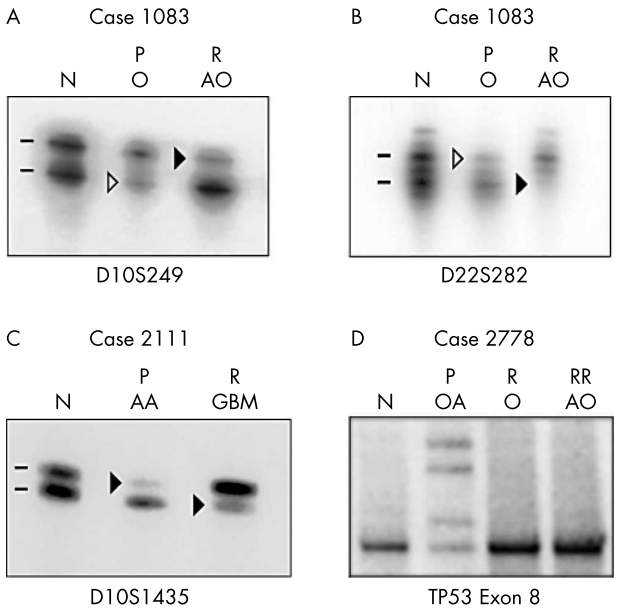

Figure 1 Cases with apparent genetic heterogeneity in the primary tumour. (A, B) Microsatellite analysis of the primary (P) and recurrent (R) tumour of case 1083. (A) Diminished intensity of the bottom allele of D10S249 in the primary tumour (open arrowhead) and loss of the top allele in the recurrent tumour (filled arrowhead). (B) Diminished intensity of the top allele of D22S282 in the primary tumour and loss of the bottom allele in the recurrent tumour. (C) Microsatellite analysis of the primary and recurrent tumour of case 2111. Loss of the top allele of D10S1435 in the primary tumour and loss of the bottom allele in the recurrent tumour. (D) DGGE analysis of exon 8 of *TP53* of the primary tumour and first and second recurrent tumour of case 2778. Presence of the *TP53* mutation in the primary tumour, absence in the first and second recurrent tumour. N, normal DNA from corresponding, leukocytes. See table 1 for tumour abbreviations.

previous study.[3] The 40 cases were subdivided in 14 cases with a same lower grade recurrence, 12 cases with a (variant) glioblastoma recurrence and 14 cases with a higher grade recurrence. The combined data are listed in table 1. As can be concluded from this table, in many cases the investigated genetic changes proved to be already present in the primary tumour and retained in the recurrence. Cases with additional genetic changes in the first or second recurrence were found and these are shown in bold. Apparent discrepancies between the genetic changes in the primary tumour and the corresponding recurrent tumour were noted in three cases of low grade glioma with a higher grade recurrence. Case 1083 displayed allelic imbalance with apparent reduction in intensity of one allele in the primary tumour for markers in 10p15 and 22q13, whereas the other allele was almost completely lost in the recurrent tumour (fig 1A, B, table 2). In case 2111, the lost allele of markers in 10p15 seems to be different in the primary tumour and the recurrent tumour. (fig 1C, table 2). Case 2778 had a *TP53* mutation in exon 8 in the primary tumour, which was no longer present in the first or second recurrent tumour (fig 1D).

DISCUSSION

Genetic heterogeneity in the primary tumour

Tumours are thought to develop by the temporal acquisition of genetic changes that confer growth advantage to a cell.[11] In a primary tumour, this clonal evolution may result in the development of variant sublines with different sets of genetic changes. The apparent discrepancies in genetic changes between primary and recurrent tumour that we noted might be explained by the presence of more than one subclone in the primary tumour. In case 1083, an early event would be inactivation of *TP53* by mutation and deletion, since both genetic changes were already present in the primary tumour (table 1). In a subsequent step, two subclones would have developed in which one or the other—10p15 or 22q13—chromosomal region was lost. Since both subclones were probably present, their mixed opposite allelic losses could have resulted in the allelic imbalances noted for this primary tumour (fig 1A, B, table 2). Outgrowth of one subclone with additional LOH at 1p36 and *DMBT1* would then have resulted in the recurrent tumour with almost complete allelic loss at 10p15 and 22q13. The apparently opposite allelic losses for 10p15 markers in primary and recurrent tumour of case 2111 might be explained by assuming that the recurrent tumour was the outgrowth of a subclone that was underrepresented in the primary tumour. In case 2778, the primary tumour was an oligoastrocytoma with clear oligodendroglial and astrocytic elements (not shown). Others have analysed the two components of biphasic oligoastrocytomas and found mutation and overexpression of *TP53* in the astrocytic component only.[12] Therefore, in our case it might be that the recurrent tumours, which were oligodendrogliomas, were outgrowths of the oligodendroglial component of the primary tumour without *TP53* mutation. We conclude that lower grade primary gliomas might be genetically heterogeneous, permitting outgrowth to a higher grade recurrence of a subclone that was not dominantly present in the primary tumour.

Additional genetic changes in the recurrent tumour

The total number of additional genetic changes acquired during glioma recurrence was considerably higher for the group of lower grade gliomas with a higher grade recurrence (mean 2.0 genetic changes per case) than for the group of lower grade gliomas with a same grade recurrence (mean 0.6) or the group of glioblastoma recurrences (mean 0.8). In accordance with earlier reports,[13 14] we found a remarkable high frequency of *TP53* mutation (93%) in the primary tumour of the group of higher grade recurrence cases. Inactivation of *TP53* is supposed to induce genetic instability, including specific chromosomal deletions.[15] The additional genetic changes acquired during glioma recurrence might be a measure for the latter. However, analysing all 40 cases of glioma recurrence, we did not find a statistically significant association between the presence of *TP53* mutation in the primary tumour and the presence of additional genetic

changes in the recurrence (*TP53* mutation yes *v* no, p = 0.21, χ^2). Therefore, it is unlikely that *TP53* mutation in the primary tumour is the major cause of the increased accumulation of genetic changes in the higher grade recurrence cases. It remains to be determined what other factors induce the increased accumulation of genetic changes in the latter cases.

Genetic changes in operable recurrent glioblastomas

EFGR amplification in the primary glioblastoma—that is, a glioblastoma at first presentation, was present in only one of 12 (8%) glioblastoma recurrence cases (table 1). This is remarkable considering that unselected primary glioblastomas display *EGFR* amplification in 34% of cases.[10] It has to be emphasised that all glioblastomas recur but that in only a small percentage of cases the recurrence is operated depending on the presence of prognostically more favourable clinical indices, such as high Karnofsky performance score, low age of the patient, and long interval between primary tumour and recurrence. Others[16] have shown that the time to glioblastoma recurrence is significantly shorter for patients with *EGFR* amplification than for those without. These data would indicate that primary glioblastomas with *EGFR* amplification have a small chance to recur as an operable tumour. In our series of glioblastoma recurrences, four cases (33%) displayed complete LOH 10, and five cases (42%) had *TP53* mutation in the primary tumour. This is considerably less frequent for LOH 10 and more frequent for *TP53* mutation than reported for unselected primary glioblastomas (about 70% and 20%, respectively).[10] Infrequent *EGFR* amplification and frequent *TP53* mutation are the genetic hallmarks of secondary glioblastomas—that is, glioblastomas that originate from a lower grade precursor lesion.[17 18] It seems that primary glioblastomas that recur as operable tumours genetically resemble secondary glioblastomas. Secondary glioblastomas typically develop in young patients (less than 45 years of age) and primary glioblastomas in older patients (mean age of 55 years).[1] Indeed, the mean age of the patients in our glioblastoma recurrence group was 45.8 years (table 1), which corresponds more to the age at which patients develop a secondary glioblastoma. Finally, the glioblastomas that recurred as an operable tumour were glioblastomas at first presentation. However, it cannot be excluded that these primary tumours developed from an undetected precursor lesion and were in fact secondary glioblastomas.

In conclusion, unselected primary glioblastomas—that is, with or without an operable recurrence, display *EGFR* amplification in 34% of cases.[10] We found a low frequency of *EGFR* amplification (8%) in our series of primary glioblastomas that recur as an operable tumour. Taken together, this would indicate that primary glioblastomas with *EGFR* amplification would have a small chance to recur as an operable tumour.

ACKNOWLEDGEMENTS

We thank A Ararou, P W A Cornelissen, and S Redeker for expert technical assistance. This work was supported by the Dutch Cancer Society, grant number UVA 2001–2561.

Authors' affiliations

T J M Hulsebos, Department of Human Genetics, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
D Troost, Department of Neuropathology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
S Leenstra, Department of Neurosurgery, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Competing interests: none declared

REFERENCES

1 **Kleihues P**, Cavenee WK, eds. *Pathology and genetics of tumours of the nervous system*. Lyon: IARC Press, 2000.
2 **Ware ML**, Berger MS, Binder DK. Molecular biology of glioma tumorigenesis. *Histol Histopathol* 2003;**18**:207–16.
3 **Hulsebos TJM**, Oskam NT, Troost D, *et al*. Dynamics of genetic alterations associated with glioma recurrence. *Genes Chromosomes Cancer* 1998;**23**:153–8.
4 **Ino Y**, Silver JS, Blazejewski L, *et al*. Common regions of deletion on chromosome 22q12.3–q13.1 and 22q13.2 in human astrocytomas appear related to malignancy grade. *J Neuropathol Exp Neurol* 1999;**58**:881–5.
5 **Oskam NT**, Bijleveld EH, Hulsebos TJM. A region of common deletion in 22q13.3 in human glioma associated with astrocytoma progression. *Int J Cancer* 2000;**85**:336–9.
6 **Hamelin R**, Jego N, Laurent-Puig P, *et al*. Efficient screening of p53 mutations by denaturing gradient gel electrophoresis in colorectal tumors. *Oncogene* 1993;**8**:2213–20.
7 **Leenstra S**, Oskam NT, Bijleveld EH, *et al*. Genetic sub-types of human malignant astrocytoma correlate with survival. *Int J Cancer* 1998;**79**:159–65.
8 **Cairncross JG**, Ueki K, Zlatescu MC, *et al*. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J Natl Cancer Inst* 1998;**90**:1473–9.
9 **Schmidt MC**, Antweiler S, Urban N, *et al*. Impact of genotype and morphology on the prognosis of glioblastoma. *J Neuropathol Exp Neurol* 2002;**61**:321–8.
10 **Von Deimling A**, Fimmers R, Schmidt MC, *et al*. Comprehensive allelotype and genetic analysis of 466 human nervous system tumors. *J Neuropathol Exp Neurol* 2000;**59**:544–58.
11 **Nowell P**. The clonal evolution of tumor cell populations. *Science* 1976;**194**:23–8.
12 **Dong ZQ**, Pang JCS, Tong CY, *et al*. Clonality of oligoastrocytomas. *Hum Pathol* 2002;**33**:528–35.
13 **Reifenberger J**, Ring GU, Gies U, *et al*. Analysis of p53 mutation and epidermal growth factor receptor amplification in recurrent gliomas with malignant progression. *J Neuropathol Exp Neurol* 1996;**55**:822–31.
14 **Ishii N**, Tada M, Hamou MF, *et al*. Cells with TP53 mutations in low grade astrocytic tumors evolve clonally to malignancy and are an unfavorable prognostic factor. *Oncogene* 1999;**18**:5870–8.
15 **Jain AN**, Chin K, Borresen-Dale AL, *et al*. Quantitative analysis of chromosomal CGH in human breast tumors associates copy number abnormalities with p53 status and patient survival. *Proc Natl Acad Sci USA* 2001;**98**:7952–7.
16 **Schlegel J**, Merdes A, Stumm G, *et al*. Amplification of the epidermal-growth-factor-receptor gene correlates with different growth behaviour in human glioblastoma. *Int J Cancer* 1994;**56**:72–7.
17 **Von Deimling A**, von Ammon K, Schoenfeld D, *et al*. Subsets of glioblastoma multiforme defined by molecular genetic analysis. *Brain Pathol* 1993;**3**:19–25.
18 **Kleihues P**, Ohgaki H. Primary and secondary glioblastoma: from concept to clinical diagnosis. *Neuro-oncol* 1999;**1**:44–51.