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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

MGMT promoter hypermethylation is a frequent, early, and consistent event in astrocytoma progression, and not correlated with *TP53* mutation

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Abstract Hypermethylation of the *MGMT* gene promoter and mutation of the *TP53* tumor-suppressor gene are frequently present in diffuse astrocytomas. However, there is only anecdotal information about *MGMT* methylation status and *TP53* mutations during progression of low-grade diffuse astrocytoma (AII) to anaplastic astrocytoma (AIII) and secondary glioblastoma (sGB). In this study biopsy specimens from 51 patients with astrocytic tumors with radiologically proved progression from low to high-grade malignancy were investigated for the presence and consistency of *MGMT* promoter hypermethylation and *TP53* mutations. For 27 patients biopsy samples both of primary tumors and their recurrences were available. For the other 24 patients histology of either the low-grade lesion or the high-grade recurrence was available. It was found that *MGMT* promoter hypermethylation and *TP53* mutations are both frequent and early events in the progression of astrocytomas and that their status is consistent over time. No correlation was found between *MGMT* methylation status and the presence of *TP53* mutations. In addition, no correlation was found between *MGMT* promoter hypermethylation and the type of *TP53* mutations. These results argue against the putative *TP53* G:C>A:T transition mutations suggested to occur preferentially in *MGMT* hypermethylated tumors.

Keywords Diffuse astrocytoma WHO grade II · Anaplastic astrocytoma WHO grade III · *TP53* mutation · *MGMT* promoter hypermethylation · G:C>A:T transition mutations

Introduction

Low-grade diffuse astrocytomas (WHO grade II; AII) are slowly growing tumors with a peak incidence in young adults. They diffusely infiltrate the normal brain and have an intrinsic tendency to progress to greater malignancy, i.e. anaplastic astrocytoma (WHO grade III; AIII) and secondary glioblastoma (WHO grade IV; sGB). Currently available treatment strategies, for example tumor resection, radiotherapy, and/or chemotherapy are only partially effective and most patients develop recurrent or progressive disease. For development of new treatment strategies, identification of genetic alterations associated with pathogenesis, progression, and treatment response of these slow-growing tumors is necessary.

The tumor-suppressor gene *TP53* on chromosome 17p13.1 encodes a key transcription factor involved in several cellular mechanisms including growth arrest, DNA repair, and induction of apoptosis. Mutations in the *TP53* gene have been reported as early and frequent events in sGB and their precursor lesions [1, 2] whereas de-novo

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primary glioblastomas reportedly lack *TP53* mutations or acquire them late in tumorigenesis [3, 4]. However, the presence of *TP53* mutations in the progression of AII to AIII and sGB has mostly been determined in investigations of series of astrocytic tumors with different grades of malignancy without following individual tumors for their tumorigenesis [5, 6]. In some but not all studies on AII, the *TP53* mutation was found to be an independent unfavorable predictor of survival and/or malignant transformation [3, 7–9]. Other genetic aberrations in the *TP53* pathway, for example *MDM2* amplification and $p14^{ARF}$ deletion or hypermethylation are also frequently observed in low-grade diffuse astrocytomas [7, 10, 11].

Over the last decade, epigenetic silencing of the O^6-methylguanine-methyltransferase (*MGMT*) gene by promoter hypermethylation has been found to be associated with chemosensitivity of a variety of tumor types, including gliomas [12–16]. The *MGMT* gene is located on chromosome band 10q26 and encodes for O^6-alkylguanine-DNA-alkyltransferase (AGT), a repair enzyme that removes promutagenic alkylating DNA adducts from the O^6 position of guanine, an important target of alkylating and methylating agents. Lack of *MGMT* repair capacity contributes to the genesis and progression of human cancers, because it leads to the accumulation of DNA mutations and chromosomal instability [12, 14, 17]. During chemotherapy with alkylating and methylating agents the primary cell mechanism inducing chemoresistence is the demethylating activity of AGT, which removes the alkyl adducts. The absence of *MGMT* activity can therefore be used as a predictor of response for patients treated with alkylating chemotherapeutic agents. High-grade gliomas, in particular glioblastomas, with hypermethylated *MGMT* promoters proved to be more sensitive to chemotherapeutic agents, including temozolomide (TMZ), resulting in an overall survival benefit for these patients [13, 15, 18–20].

Apart from the predictive value, the *MGMT* promoter hypermethylation status has been associated with shorter progression-free survival in AII [21, 22]. In several reports an association between *MGMT* promoter hypermethylation and an increased frequency of *TP53* mutations, with predominance of G>A or C>T transitions (G:C>A:T transition mutations), especially at CpG sites, has been mentioned [11, 21, 23–25]. Further, in a recent population-based study of glioblastomas a higher frequency of *TP53* G:C>A:T transition mutations in tumors with *MGMT* promoter hypermethylation (25%) than in glioblastomas without *MGMT* methylation (16%, $P = 0.0385$) was reported [26]. However, information about *MGMT* promoter hypermethylation or *TP53* mutation during astrocytoma progression is sparse [11, 23]. The objective of this study was to investigate the association of *MGMT* promoter methylation status and *TP53* mutations and to determine the consistency of these molecular characteristics over time, in a series of diffuse astrocytomas and their recurrences.

Materials and methods

Patients and tumor samples

Archival routine formalin-fixed and paraffin-embedded (FFPE) tumor tissues were collected from 51 patients diagnosed with:

(1) an AII at the time of the first operation; or
(2) a histopathology of high-grade astrocytic tumor after a wait and see period of at least 1.5 years and a lesion on MR scan without contrast enhancement (clinically regarded as an original low-grade astrocytic tumor).

Glioma recurrences were diagnosed by contrast enhancement on MR scans, with or without clinical symptoms, and/or histopathology of tissue biopsies. For 27 patients tumor tissue was available both from the first operation and from the operation for the recurrence. For 17 patients only tumor tissue from the first operation was available and for seven patients only tissue from the second surgery was available (Table 1). Hematoxylin and eosin (H&E)-stained tissue sections from all tumors were reviewed by an independent neuropathologist (JMK) in accordance with the latest WHO guidelines for typing and grading [27].

All patients received radiation therapy after surgery for the primary tumor. Four of the 27 patients (Table 1; cases 21, 22, 29, and 32) were treated with alkylating chemotherapy (procarbazine, lomustine, and vincristine) between the operations for the primary and recurrent tumors. All patients were treated with temozolomide after the operation for the recurrent tumor. By the end of this study all patients had died. The median follow-up time was 65.5 months.

The archival tissue samples were used in accordance with the code of conduct for appropriate secondary use of tissue: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

DNA extraction

H&E-stained sections from the available FFPE tissue blocks were used by the neuropathologist (JMK) to select parts for DNA extraction. DNA was isolated by standard procedures from selected tissue fragments containing a high percentage tumor cells and, when possible, from fragments of normal tissue [28].

Table 1 Results from histopathological review and molecular analysis

Case no.	Sex	Age	P/R	Interval (months)	Intervening treatment	Histology	p53 expression % of cells	Mutation analysis of *TP53*	LOH of the *TP53* locus [b]	*MGMT* status
Primary only ($n = 17$)										
1	M	39	P			AII	80	Exon 6, c.13419A>G, p.Tyr220Cys	Yes	Hypermethylated
2	F	29	P			AII	60	Exon 4, c.12243_12260dupTACGGTTTCCGTCTGGGC, p.Tyr107_Gly112dup	No	Hypermethylated
3	F	39	P			AIII	60	Exon 4, c.12253G>T, p.ArgR110Leu/Exon 8, c.14486C>T, p.Arg273Cys	No	Hypermethylated
4	M	38	P			AII	60	Exon 5, c.13107T>G, p.Val143Gly	Yes	Hypermethylated
5	F	40	P			AII	30	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Unmethylated
6	M	38	P			AII	40	Exon 8, c.14496C>A, p.Ala276Asp	Yes	Hypermethylated
7	M	47	P			AII	30	Exon 8, c.14470_14475dupAACAGC, p.Asn268_Ser269dup	No	Hypermethylated
8	M	51	P			AII	Neg.	Exon 5, c.13229delG, p.Asp184 fs	No	Hypermethylated
9	F	33	P			AII	30	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Unmethylated
10	M	32	P			AIII	20	No data	Yes	Hypermethylated
11	F	34	P			AII	70	No data	Yes	No data
12	M	36	P			AII	20	Exon 8, c.14487G>A, p.Arg273His	No	Hypermethylated
13	F	59	P			AII	60	Wt	Yes	Unmethylated
14	M	25	P			AII	>10	Exon 5, c.13203G>A, p.Arg175His/Exon 8, c.14513C>T, p.Arg282Trp	No	Hypermethylated
15	M	38	P			AIII	80	Exon 6, c.13329C>G, p.Pro190Arg	Yes	Hypermethylated
16	M	43	P			AII	80	Wt	Yes	Hypermethylated
17	M	39	P			AII	50	Exon 7, c.14061G>C, p.Gly245Ala	Yes	Unmethylated
Primary and recurrence ($n = 27$)										
18[a]	M	35	P	4	RT	AIII	30	Exon 5, c.13107T>G, p.Val143Gly	Yes (reverse loss)	Hypermethylated
			R			GBM	40	Wt		Unmethylated
19	F	49	P	8	RT	AIII	40	Exon 4, c.12253G>C, p.ArgR110Pro/Exon 5, c.13152G>A, p.Arg158His	No	Hypermethylated
			R			GBM	30	Exon 4, c.12253G>C, p.ArgR110Pro/Exon 5, c.13152G>A, p.Arg158His	No	Hypermethylated
20	M	41	P	12	RT	GBM	>80	Exon 5, c.13152G>C, p.Arg158Pro	No	Unmethylated
			R			GBM	>80	Exon 5, c.13152G>C, p.Arg158Pro	Yes	Unmethylated
21	F	48	P	12	RT + PCV	AII	<10	Wt	No	Hypermethylated
			R			GBM	80	Wt	No	Hypermethylated
22	M	34	P	13	RT + PCV	AII	20	Exon 5, c.13149T>G, p.Val157Gly	Yes	Unmethylated
			R			AII	70	Exon 5, c.13149T>G, p.Val157Gly	Yes	Unmethylated
23	F	56	P	17	RT	GBM	>80	Exon 5, c.13203G>A, p.Arg175His	No	Unmethylated
			R			GBM	>80	Exon 5, c.13203G>A, p.Arg175His	No	Unmethylated

Table 1 continued

Case no.	Sex	Age	P/R	Interval (months)	Intervening treatment	Histology	p53 expression % of cells	Mutation analysis of *TP53*	LOH of the *TP53* locus [b]	*MGMT* status
24	F	36	P	18	RT	AII	60	No data	Yes	No data
			R			AII	60	No data	Yes	No data
25	M	54	P	23	RT	AIII	>80	Exon 5, c.13203G>A, p.Arg175His	Yes	Hypermethylated
			R			GBM	>90	Exon 5, c.13203G>A, p.Arg175His	Yes	Hypermethylated
26	F	31	P	23	RT	AII	>80	Wt	Yes	Hypermethylated
			R			GBM	>80	Wt	Yes	Hypermethylated
27	F	55	P	25	RT	AII	<10	Wt	No	Unmethylated
			R			AII	<10	Wt	No	Unmethylated
28	M	44	P	25	RT	AII	>10	Exon 7, c.14057G>T, p.Gly244Cys	Yes	Unmethylated
			R			AIII	>80	Exon 7, c.14057G>T, p.Gly244Cys	Yes	Unmethylated
29	M	31	P	26	RT + PCV	AII	20	Exon 5, c.13107T>G, p.Val143Gly	Yes	Hypermethylated
			R			OIII	>90	Exon 5, c.13107T>G, p.Val143Gly	Yes	Hypermethylated
30	F	34	P	37	RT	OAIII [c]	70	Exon 6, c.13419A>G, p.Tyr220Cys/Exon 8, c.14487G>A, p.Arg273His	No	Unmethylated
			R			OAII [c]	60	Exon 6, c.13419A>G, p.Tyr220Cys/Exon 8, c.14487G>A, p.Arg273His	No	Unmethylated
31	M	28	P	37	RT	AII	30	Exon 6, c.13419A>G, p.Tyr220Cys	Yes	No data
			R			AIII	80	Exon 6, c.13419A>G, p.Tyr220Cys	Yes	Unmethylated
32	M	55	P	40	RT + PCV	AII	Neg.	Exon 5, c.13157_13161delATGGC, p.Met160 fs	Yes	Unmethylated
			R			GBM	Neg.	Exon 5, c.13157_13161delATGGC, p.Met160 fs	Yes	Unmethylated
33	M	36	P	43	RT	AII	30	Exon 8, c.14487G>A, p.Arg273His	Yes	Hypermethylated
			R			AIII	>80	Exon 8, c.14487G>A, p.Arg273His	Yes	Unmethylated
34[a]	F	29	P	43	RT	AII	20	Exon 7, c.14063A>G, p.Met246Val	Yes	Unmethylated
			R			GBM	60	Exon 8, c.14483G>T, p.Val272Leu	Yes	Hypermethylated
35	F	34	P	47	RT	AII	30	Exon 5, c.13197T>A, p.Val173Glu/Exon 7, c.14070G>A, p.Arg248Gln	No	Hypermethylated
			R			GBM	>80	Exon 5, c.13197T>A, p.Val173Glu/Exon 7, c.14070G>A, p.Arg248Gln	No	Hypermethylated
36[a]	F	33	P	56	RT	AII	30	Wt	Yes (reverse loss)	No data
			R			GBM	>80	Exon 6, c.13419A>G, p.Tyr220Cys		Unmethylated
37	F	39	P	62	RT	AIII	60	Exon 5, c.13056A>G, p.Tyr126Cys	No	Hypermethylated
			R			OAIII	60	Exon 5, c.13056A>G, p.Tyr126Cys	No	Hypermethylated
38	M	39	P	73	RT	AII	>10	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Unmethylated
			R			OAIII	80	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Hypermethylated
39	M	41	P	74	RT	AII	50	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Unmethylated
			R			AII	80	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Unmethylated

Table 1 continued

Case no.	Sex	Age	P/R	Interval (months)	Intervening treatment	Histology	p53 expression % of cells	Mutation analysis of *TP53*	LOH of the *TP53* locus [b]	*MGMT* status
40	M	55	P	85	RT	AII	<10	Wt	No	Hypermethylated
			R			GBM	80	Wt	Yes	Hypermethylated
41	F	37	P	95	RT	AII	60	Wt	Yes	Hypermethylated
			R			GBM	80	Wt	Yes	No data
42	F	38	P	96	RT	AII	80	Exon 6, c.13418T>C, p.Tyr220His	Yes	Hypermethylated
			R			AII	>10	Exon 6, c.13418T>C, p.Tyr220His	Yes	Hypermethylated
43	M	46	P	146	RT	AII	30	Wt	No	Unmethylated
			R			AII	30	Exon 9, c.14719_14735dupAAGAAACCACTGG, p.Glu326 fs	Yes	Unmethylated
44[a]	M	37	P	160	RT	AII	80	Exon 9, c.14686C>T, p.Pro309Ser	Yes (reverse loss)	Unmethylated
			R			AII	60	Exon 9, c.14710C>T, p.Gln317Stop		Unmethylated
Recurrence only ($n = 7$)										
45	M	43	R			AII	<10	Wt	No	Unmethylated
46	M	43	R			AII	20	Wt	No	Hypermethylated
47	M	27	R			GBM	>90	Exon 5, c.13203G>A, p.Arg175His	Yes	Hypermethylated
48	M	32	R			AIII	70	Exon 8, c.14486C>T, p.Arg273Cys	Yes	Unmethylated
49	M	49	R			GBM	>80	Exon 8, c.14486C>T, p.Arg273Cys	No	Hypermethylated
50	F	44	R			AII	20	Wt	No	No data
51	F	37	R			GBM	>10	Exon 5, c.13203G>A, p.Arg175His	Yes	No data

Summary of results from 51 patients diagnosed with a recurrent astrocytic tumor with a clinical history of primary diffuse astrocytoma (WHO grade II)

For 27 patients tumor specimens from primary and recurrent tumor were available; for 17 patients only biopsy material from the first operation was available; and for seven patients only material from the second surgery was available. After molecular investigation of the 27 cases with paired primary tumors and recurrences (cases 18–44), four cases with different *TP53* mutations and/or LOH pattern were considered as two, clonally independent, entities (cases 18, 34, 36, and 44)

P, primary; R, recurrence; RT, radiotherapy; PCV, procarbazine, lomustine, and vincristine; AII, low-grade astrocytoma WHO grade II; AIII, anaplastic astrocytoma WHO grade III; GBM, glioblastoma multiforme; OIII, anaplastic oligodendroglioma; OAII, oligo-astrocytoma; OAIII, anaplastic oligo-astrocytoma; Wt, wild-type

[a] Cases with two clonally unrelated tumors, on the basis of the molecular results

[b] Paired tumors both yes: identical LOH pattern

[c] The histology of the recurrence seemed to be indicative of lower malignancy grade than the primary tumor; this is attributed to sampling error in the recurrence (case 30)

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) for *MGMT*

The MS-MLPA probe mix used (ME011; MRC Holland, Amsterdam, The Netherlands) includes 21 probe sequences containing an *Hha*I recognition site, which yields information about the methylation status of the target sequences. In addition, eight control probe sequences not affected by the methylation-sensitive restriction enzyme *Hha*I digestion are used. The probes containing an *Hha*I recognition site should only generate a signal if the DNA target is methylated and cannot be digested. The probe mix was developed to detect CpG island methylation of six mismatch repair genes and includes three specific probes for semiquantitative hypermethylation detection of the *MGMT* promoter region. This MS-MLPA assay for *MGMT* promoter hypermethylation of gliomas was performed as described by Jeuken et al., with minor modifications [29]. For fragment analysis, PCR products were separated by capillary gel electrophoresis (ABI Prism 3130xl; Applied Biosystems, Foster City, CA, USA) and quantified by use of GeneMarker software version 1.7 (SoftGenetics, State College, PA, USA).

MS-MLPA data analysis

The MS-MLPA results were normalized by dividing the peak height for each *MGMT* probe signal by the mean peak height for the eight control fragments within the same sample. To estimate the fraction of methylated *MGMT* promoter DNA, normalized values of each *MGMT* probe of digested DNA samples were divided by normalized values of corresponding undigested DNAs. Methylation analyses were performed in duplicate and the average ratios of both experiments for each probe were calculated. For the promoter hypermethylation detection of *MGMT*, we used the $MGMT_2$ probe from the probe mix only. This probe is located within the widely used CpG island region for *MGMT* promoter hypermethylation detection by methylation-specific PCR (MS-PCR), and hypermethylation of this region has been found to be an independent predictor of the response of gliomas to TMZ [13, 15, 19, 20].

Preliminary comparison of the widely used MS-PCR and the $MGMT_2$ probe analyses demonstrated concordant results (not shown). Also, in our hands, the $MGMT_2$ probe also resulted in better assay reproducibility (Spearman's rho 0.658, $P < 0.001$) than the two other *MGMT* probes ($MGMT_1$ probe Spearman's rho 0.448; $P = 0.032$ and $MGMT_3$ probe Spearman's rho 0.431; $P = 0.040$). Cell lines were used as positive (TE-4, OEC33, FLO-1, SW620) or negative (TE-1, TE-5, TE-13, TE-14, SK-GT-2, BE-3, ESO51) controls for evaluation of *MGMT* promoter methylation. When the *MGMT* digested/*MGMT* undigested ratio of probe $MGMT_2$ is below 0.30 the *MGMT* promoter fragment is regarded as non-methylated, as described previously for MS-MLPA assays [30, 31].

Mutation analysis of *TP53*

Tumor samples from each patient were screened for *TP53* mutations in exons 4–9, including intron–exon boundaries, by polymerase chain reaction (PCR) followed by bi-directional direct DNA sequencing using the M13-tailed primer method. Intronic primer sets for PCR amplification are shown in Table 2A. PCR products were generated in a 15-µl reaction mixture containing 1.0 µl DNA solution, 10 µmol of each primer, 25 mM $MgCl_2$, 10 mM dNTPs, and 1U Taq polymerase (Promega, Madison, WI, USA). The PCR reaction was performed using a thermocycler (Biometra, Göttingen, Germany) with an initial denaturing step (95°C) for 3 min, followed by 35 cycles consisting of denaturing (95°C) for 30 s, annealing (60°C) for 45 s, and extension (72°C) for 45 s. After the final cycle, an extension period of 10 min at 72°C was performed. The PCR products (5 µl) were digested with 1 µl exonuclease I (ExoSAP-IT; USB, Cleveland, OH, USA) at 37°C for 30 min followed by inactivation of the enzyme at 80°C for 15 min. The PCR products were sequenced on an ABI Prism 3130xl genetic analyzer using the ABI Prism *BigDye* Terminator v3.1 kit (both from Applied Biosystems). Samples were analyzed by use of Mutation Surveyor software package version 3.2 (SoftGenetics, State College, PA, USA) and compared with the public sequence in GenBank (NM_000546).

Loss of heterozygosity (LOH) analysis of the *TP53* locus (17p13)

Five highly polymorphic microsatellite markers located on chromosome 17p13 (D17S1353, D17S1866, D17S1566, D17S786, and D17S520) were used for LOH analysis. Microsatellites were amplified with fluorescence-labeled forward and unlabeled reverse primers (Table 2B). Because normal DNA from non-neoplastic tissues was not available for most of the tumor samples, allelic losses were determined by analysis of the allelic patterns and by integration of data from multiple highly polymorphic markers, as described elsewhere [32]. LOH analysis was performed using the ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) and the GeneMarker software package version 1.7 (SoftGenetics).

Immunohistochemistry (IHC) for p53

IHC for p53 was performed on 4-µm sections of routine FFPE tissues. Briefly, sections were deparaffinized in

Table 2 Primer sets for *TP53* mutation analysis and LOH analysis of the *TP53* Locus

Exon	Size (bp)[a]	Forward primer	Reverse primer
A Nucleotide sequences of primers used for *TP53* mutation analysis			
Exon 4A	300	5′-CTGGTAAGGACAAGGGTTGG-3′	5′-GATGACAGGGGCCAGGAG-3′
Exon 4B	249	5′-AGATGAAGCTCCCAGAATGC-3′	5′-GATACGGCCAGGCATTGAAG-3′
Exon 5A	222	5′-TGCTGCCGTCTTCCAGTTGC-3′	5′-CTCACAACCTCCGTCATGTG-3′
Exon 5B	197	5′-CAGCTGTGGGTTGATTCCAC-3′	5′-TGAGGAATCAGAGGCCTG-3′
Exon 6	263	5′-TCAGATAGCGATGGTGAGCA-3′	5′-GCCACTGACAACCACCCTTA-3′
Exon 7	215	5′-CGCACTGGCCTCATCTTG-3′	5′-AGGGGTCAGAGGCAAGCAGA-3′
Exon 8	248	5′-GGGACAGGTAGGACCTGATTT-3′	5′-GCATAACTGCACCCTTGGTC-3′
Exon 9	232	5′-GGAGACCAAGGGTGCAGTTA-3′	5′-CCCCAATTGCAGGTAAAACA-3′
B Nucleotide sequences of primers used for LOH analysis of the *TP53* Locus			
D17S520	142	5′-GGAGAAAGTGATACAAGGGA-3′	5′-TAGTTAGATTAATACCCACC-3′
D17S786	167	5′-TACAGGGATAGGTAGCCGAG-3′	5′-GCTGTGAGTCCCAAAAGAGG-3′
D17S1353	122	5′-ATTCCCACTGCCACTCCTTG-3′	5′-CAGCTGAGGGATACTATTCAG-3′
D17S1566	189	5′-AAAGATCCTTATTGCCACTTTACTG-3′	5′-CTCTTACCTTGCTGGTGAGATTG-3′
D17S1866	175	5′-TGGATTCTGTAGTCCCAGG-3′	5′-GGTTCAAAGACAACTCCCC-3′

[a] Amplicon sizes based on the UCSC Genome Browser (http://genome.ucsc.edu)

xylene and hydrated through descending ethanol gradients. Endogenous peroxidase was inactivated by treatment with 3% H_2O_2 in phosphate-buffered saline (PBS). Antigen retrieval was accomplished by boiling the sections for 15 min in 10 mmol/l Tris–EDTA, pH 9.0 in a microwave oven. The sections were incubated for 1 h at room temperature with mouse monoclonal antibody DO-7 against p53 protein (Dako, Glostrup, Denmark) at 1:50 dilution. After rinsing in PBS, immunoreactivity was visualized using the Envision kit (Dako). The sections were subsequently counterstained with Mayer hematoxylin. Although the DO-7 antibody binds to both normal and mutant p53 protein, in general, normal levels of wild-type p53 protein are too low to be detected by immunohistochemistry. In each section, the number of clearly positive cells out of 1000 cells was counted and >10% was regarded as positive [33].

Statistical analysis

Correlation coefficients were calculated by use of Student's *t*-test and Fisher's exact test. Data were regarded as statistically significant at $P < 0.05$.

Results

The results from histopathological review and molecular analysis are summarized in Table 1. After molecular investigation of the 27 cases of paired primary tumors and recurrences (Table 1, cases 18–44) four cases with different *TP53* mutations and/or reverse LOH patterns were regarded as two clonally independent entities (cases 18, 34, 36, and 44). The strongest indication of clonally independent entities is the finding of specific molecular aberrations in the primary tumors which are not found in the recurrences. In case 43 neither a *TP53* mutation nor LOH was found in the primary tumor whereas in the recurrence a *TP53* mutation and LOH were detected. This could indicate clonally independent tumors. However, because no molecular aberrations were detected in the primary tumor and because of the long period of time between occurrence of the primary lesion and recurrence (146 months) we regard case 43 as more likely to be clonally related tumors with generation and clonal selection of the aberrations during the long time interval between both tumors.

Patients and tumor histology

According to central review, 34 of 44 (77%) primary tumors were diffuse astrocytomas of WHO grade II (AII); 6 of 44 (14%) were anaplastic astrocytomas (AIII), and 3 of 44 (7%) were glioblastoma (GB). One primary tumor was diagnosed as an anaplastic mixed oligoastrocytoma (OAIII). Of the recurrent tumors 10 of 34 (29%) were still AII; 5 of 34 (15%) had progressed to AIII; and 15 of 34 (44%) became secondary glioblastomas (sGBs). Two recurrent tumors were diagnosed as anaplastic mixed oligoastrocytomas (OAIII), one as anaplastic oligodendroglioma (OIII), and one as low-grade oligoastrocytoma (OAII). In all tumors with an oligodendroglial component found by

central review, loss of chromosome 1p and/or 19q was excluded in these cases (results not shown).

Of the 27 cases of which both the primary and recurrent tumor were investigated, 23 cases were regarded as having clonally related tumors and four cases clonally unrelated tumors, on the basis of molecular results. Thirteen of 23 (57%) clonally related tumor pairs showed histological progression of malignancy grade. In nine cases (39%) the tumor histology had not progressed in malignancy grade. In one case the histology of the recurrence seemed to indicate lower malignancy grade than the primary tumor; this was attributed to sampling error in the recurrence (Table 1, case 30). For the 23 paired, clonally related histological samples the mean interval between surgery for the primary tumor and recurrence was 45.1 ± 35.2 months (range 8–146 months). The mean interval between surgery for primary and recurrent tumors of those cases in which the histology of the first biopsy had not progressed in malignancy grade was 50.0 months; for the tumors in which histological progression had occurred the mean interval was 41.3 months (Student's *t*-test, $P = 0.57$).

Hypermethylation analysis of the *MGMT* promoter

Seventy-eight tumor samples were available for *MGMT* promoter hypermethylation and *TP53* mutation analysis (viz.: 21 samples from patients for whom only the first specimens were available; 54 samples from 27 patients for whom both the first and second surgery tissue samples were available; and three samples from patients for whom only the second tumor specimens were available). Results showing *MGMT* promoter hypermethylation by MS-MLPA were obtained for 70 of 78 (90%) of the (routine FFPE) tumor tissues (Table 1). *MGMT* promoter hypermethylation was identified in 39 of these 70 (56%) tumor samples. There was no relationship between tumor grade (viz., AII, AIII or sGB) and methylation status (53% vs. 64% vs. 56% hypermethylated, respectively). For 20 of 23 clonally related tumor pairs *MGMT* promoter hypermethylation results were obtained from both tumors. In 18 of 20 cases (90%), primary and recurrent tumors had a similar *MGMT* promoter methylation status (nine tumor pairs hypermethylated; nine tumor pairs not methylated). For two pairs of related tumors a change of *MGMT* methylation status between the primary and the recurrent tumors was found. There was no relationship between change in *MGMT* methylation status and intervening treatment with alkylating chemotherapy. Of the four cases with clonally independent tumors, one had different *MGMT* hypermethylation status, two had identical *MGMT* hypermethylation status, and for one case no data could be obtained from the second tumor.

TP53 mutation analysis and p53 expression

TP53 exon 4–9 DNA sequence data were obtained from 74 of 78 (95%) tumor samples. Four samples had inferior DNA quality resulting in no reliable sequencing data. *TP53* mutation analysis revealed mutations in 40 of the 52 (77%) independent cases. In 35 of these 40 (88%) independent cases one mutation, and in 5 of 40 (13%) cases two *TP53* mutations within the same tumor sample were detected. *TP53* mutations were detected in 28 of 41 (68%) tumors with histological diagnosis of AII, in 10 of 10 (100%) AIII, and in 13 of 18 (72%) GB (Table 1). Mutation of *TP53* was found in 17 of 23 (74%) tumor pairs (primary tumors and their recurrences) regarded as clonally related. In three of these 17 tumor pairs two different *TP53* mutations in the same tumor sample were detected and these mutations all remained present in the recurrent tumors. Two tumor pairs had *TP53* mutations in the recurrent tumors only, and in one case *TP53* mutation was only found in the primary tumor. In two tumor pairs the mutation in the primary tumor differed from that in the recurrence. In five tumor pairs *TP53* mutations were not found in either the primary or recurrent tumor, and in one tumor pair no sequence data were obtained.

A total of 45 *TP53* mutations were detected in 40 of 55 independent tumors. Three mutations were found in *TP53* exon 4, fifteen in exon 5, six in exon 6, four in exon 7, fourteen in exon 8, and three in exon 9. A not previously reported in-frame duplication of 18 nucleotides (codon 107–112) in exon 4 was found in one patient (Table 1, case 2). Of all the *TP53* mutations identified in independent cases, 20 of 45 (44%) were G:C>A:T transition mutations; of these, 18 of 20 (90%) were located at CpG sites. Of the 25 not G:C>A:T transition mutations only three mutations (12%) were located at CpG sites. The two G:C>A:T transition mutations that were not located at CpG sites were found in exon 9, the exon of *TP53* without CpG sites.

Positive immunohistochemistry for p53 was associated with either mutation of the *TP53* gene (Fisher's exact test, $P = 0.02$) or LOH of the *TP53* locus (Fisher's exact test, $P = 0.02$) or both (Fisher's exact test, $P < 0.001$). In five of eight tumor samples without p53 expression neither *TP53* mutation nor LOH was found. The remaining three samples without p53 expression had *TP53* frameshift mutations and, in accordance with this type of mutation, p53 expression was absent.

LOH analysis of the *TP53* locus (17p13)

All 78 tumor samples in the study were informative for at least two of five microsatellite markers on chromosome 17p13. In 38 of 48 (78%) tumors with a single *TP53* mutation, LOH of the *TP53* locus was found. In all tumors

Table 3 Correlation between *MGMT* promoter hypermethylation status and *TP53* mutation status

	MGMT hypermethylated ($n = 29$)[a]	*MGMT* not hypermethylated ($n = 20$)	*P* value
TP53 mutated	21	16	0.77
G:C>A:T mutation	10	9	0.80
G>A transition	7	2	
At CpG site	*7*	*2*	
C>T transition	3	7	
At CpG site	*3*	*5*	
TP53 not mutated	7	4	

[a] Case 10: no data on *TP53* mutation could be obtained

in which two *TP53* mutations were found, no allelic loss on 17p13 was observed. LOH of the *TP53* locus was found in five out of 13 (38%) AII without *TP53* mutation and in four out of seven (57%) recurrent tumors without *TP53* mutation. The allelic loss pattern was different between three tumor pairs in which LOH was found in the recurrent tumor only. Further, reverse allelic loss of the primary and recurrent tumor was detected in three patients. One of these had a different mutation in each tumor whereas two patients had a mutation in only one of the tumor pairs.

Correlation between *MGMT* promoter hypermethylation and *TP53* mutations

There was no significant correlation between *MGMT* promoter hypermethylation and *TP53* mutation (Fisher's exact test, $P = 0.77$; Table 3). In addition, there was no significant correlation between *MGMT* promoter hypermethylation and the presence of G:C>A:T transition mutations in *TP53*. G:C>A:T transition mutations were found in 10 of 21 (48%) independent tumors with a hypermethylated *MGMT* promoter and a *TP53* mutation, and in nine of 16 (56%) *MGMT* promoter unmethylated tumors with a *TP53* mutation (Fisher's exact test, $P = 0.80$; Table 3).

Discussion

In this study we demonstrated that *MGMT* promoter hypermethylation and *TP53* aberrations are early and frequent events in the progression of astrocytomas and that both are largely consistent over time. In addition, we did not find a correlation between *MGMT* promoter methylation status and *TP53* mutations. These results argue against the putative *TP53* G:C>A:T transition mutations which have been suggested to occur preferentially in *MGMT* hypermethylated tumors.

MGMT promoter hypermethylation

No data on the *MGMT* promoter methylation status during astrocytic tumor progression have yet been reported. In this study, we showed that *MGMT* promoter hypermethylation is not limited to high-grade astrocytomas but should be regarded as an early and frequent event in primary AII and AIII, which remains present during tumor progression. *MGMT* promoter hypermethylation was found in 58% of primary AII, consistent with frequencies reported by Nakamara et al., Watanabe et al., and Nakasu et al. [11, 23, 34], who demonstrated *MGMT* promoter hypermethylation by MS-PCR or immunohistochemistry in 48, 63, and 68% of AII, respectively.

Discrepancies between results from immunohistochemistry and from PCR experiments should be taken into consideration. We found concordance in *MGMT* promoter hypermethylation status between primary and recurrent tumors in 18 of 20 (90%) clonally related tumor pairs. The remaining two cases demonstrate that *MGMT* promoter hypermethylation may change during astrocytoma progression, e.g., by reversibility of the hypermethylation, clonal selection of methylated or unmethylated subclones, or by loss of the hypermethylated *MGMT* allele. No relationship between a change in *MGMT* promoter methylation status and intervening treatment with alkylating chemotherapy was revealed. Recently, Brandes et al. reported their study on the stability of *MGMT* methylation status in a series of 38 patients with a glioblastoma and second surgery for recurrence. *MGMT* methylation status changed in 37% of the patients and more frequently in methylated than in unmethylated tumors [35].

TP53 mutations

With regard to the analyses of molecular aberrations in tumors it must be considered that, because of the general procedures used, technically only aberrations present in most of the cells from which the DNA was isolated (i.e. clonal aberrations) will be detected. Tumors are composed of neoplastic and non-neoplastic (normal) cells and it is generally accepted that the neoplastic cells, in contrast with normal cells, harbor clonal molecular aberrations. DNA isolated from a part of the tumor is composed of the DNA of the neoplastic cells and the DNA of the normal cells. These facts imply that when a specific molecular aberration is reliably identified in the DNA sample isolated from the tumor, this DNA sample is retrieved from a part of the tumor composed of a high percentage of clonal neoplastic cells. Admixture of a high percentage of normal cells or the presence of heterogeneous (with regard to the determined aberration) neoplastic cell populations would have masked the aberration and prevented its detection.

The high percentage of mutations of *TP53* (77%) detected in the astrocytic tumors in this study is in accordance with the highest frequencies reported in the literature [1, 3, 6–8]. The presence or absence of nuclear p53 expression, determined by immunohistochemistry, was highly significantly associated with the presence or absence of *TP53* aberrations. Significant correlation between p53 expression, *TP53* mutations, and *TP53* locus LOH was shown in other studies [1–3, 8]. In 13 cases with high p53 expression and/or LOH of chromosome 17p13 no *TP53* mutations were detected. This may be the result of intratumoral heterogeneity of *TP53* gene mutations [36] or *TP53* mutations escaping detection by our sequencing method (especially in cases without detected LOH, probably because of admixture of normal DNA). Yet another explanation may be that *TP53* mutations were located outside the investigated region of the gene (especially in cases with LOH). In ten cases a single *TP53* mutation was detected without concomitant *TP53* locus LOH. It may well be that in these cases LOH was probably not identified in the tumor DNA because of too much admixture of normal DNA. In addition, *TP53* LOH could be absent because of the presence of a dominant-negative *TP53* mutation in the tumors without concomitant loss of the wild type allele. In five cases two *TP53* mutations were found and in all eight tumor samples from these cases no LOH of the *TP53* locus was observed, which is more supportive of monoclonal tumor cell populations with biallelic *TP53* mutations than of dual clonality.

TP53 mutations are consistent during astrocytoma progression, because we demonstrate identical *TP53* mutation and *TP53* locus LOH status in 21 of 27 investigated pairs of primary tumor and recurrence, indicating clonal relationships between these tumor pairs. In two cases *TP53* aberrations were found in the tumor recurrences only. It may well be that the mutations were present in the first samples but not detected because the percentage of the mutation-carrying tumor cells was too low. The fact that in both primary tumors no *TP53* LOH was observed and only a low percentage of p53 positive cells was seen also indicates the presence of many normal cells in these tumor specimens. In four of 27 (15%) patients differences in *TP53* mutation and *TP53* LOH between the primary tumors and recurrences were unequivocally found. (One of these cases, case 44, is illustrated in Fig. 1) Allelotyping excluded tissue admixture in these cases. The differences between the primary and recurrent tumors may well be the result of intratumoral heterogeneity or alternatively, may be indicative of two, clonally independent, tumors. Intratumoral heterogeneity for *TP53* aberrations has been described previously for astrocytic brain tumors [36] and in the setting of systemic metastasis of glioblastomas [37]. However, in a large study on 144 biopsies from 67 patients with recurrent astrocytoma no indication of intratumoral heterogeneity was obtained [1]. In our study all eight tumor samples from the four paired cases reliably demonstrated *TP53* locus LOH with loss of the reverse alleles in three cases, indicating that the isolated DNA was retrieved from a high percentage of clonal tumor cells with little admixture of normal cells or tumor cells of another clone. In addition, in cases 34 and 44 (Table 1) the *TP53* mutation identified in the primary tumor was not found in the recurrence and, vice versa, the mutation detected in the recurrence was not present in the primary tumor. Because *TP53* mutation is an early event in astrocytoma tumorigenesis the finding of different *TP53* mutations in paired primary tumor and recurrence is supportive for dual clonality. Although intratumoral heterogeneity cannot be excluded as the cause of different *TP53* aberrations in the paired primary tumors and recurrences, we believe our results are more supportive for the presence of two, clonally unrelated, entities in these four patients.

Thirteen of the 23 clonally related pairs of primary tumor and recurrence demonstrated histologically confirmed malignant progression and all cases were consistent with regard to *TP53* aberrations (mutation, LOH and p53 expression). This result is in contrast with a study by Sarkar et al. who reported increased p53 expression upon malignant progression of astrocytic tumors. However, in that study only p53 expression was determined and no *TP53* mutation nor LOH analysis was performed. Comparable with our study, no correlation between *TP53* aberration and interval to recurrence was found [38].

Correlation between *MGMT* promoter hypermethylation and *TP53* mutation

Methylation of the *MGMT* promoter, and thus absence of AGT expression in gliomas reportedly correlates with G:C>A:T transition mutations of *TP53*, in particular at CpG sites [11, 21, 23–25]. The mechanism of the preferential occurrence of these mutations may be related to the cytosine methylation-enhanced formation of adducts at methylated CpG sites. A 5-methylcytosine adjacent to the 5′ position of an O^6-methylated guanine strongly hampers repair of this defect by AGT. Accordingly, inactivation of *MGMT* gene expression causes retention of the methylated adducts at the O^6 position of guanine. This results in spontaneous or factor-mediated deamination which converts the 5-methylcytosine into thymine. During DNA replication thymine is incorporated and subsequently a G:C to A:T transition mutation may originate at that spot. This would support the relationship between epigenetic inactivation of *MGMT* and accumulation of this mutation in *TP53* at CpG sites [23, 26].

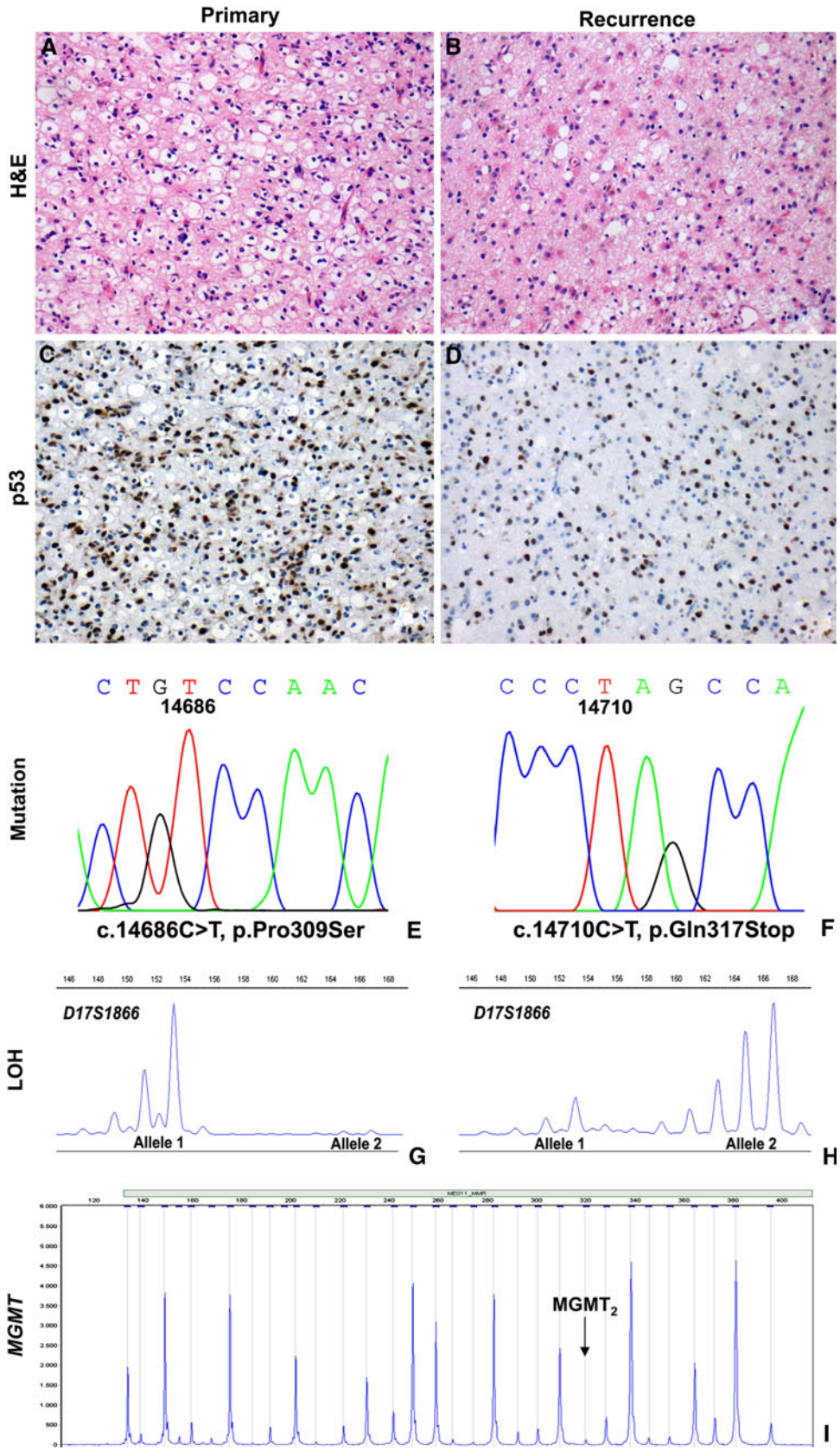

Fig. 1 Combined analysis of *TP53* and *MGMT*, case 44 primary and second tumor. **a** Diffuse astrocytoma (WHO grade II); primary tumor (the perinuclear halos are interpreted as edema of the neuropilema rather than an oligodendroglial tumor component). **b** Diffuse astrocytoma (WHO grade II); second tumor. **c**, **d** Immunohistochemistry for p53 showing many positive (brown) nuclei in both tumor samples. **e**, **f** *TP53* mutation analysis: different mutations in the primary and secondary tumors, suggestive of the presence of dual clonality. **g** Loss of heterozygosity (LOH) analysis showing loss of the larger allele in the primary tumor (microsatellite marker *D17S1866*). **h** LOH analysis showing loss of the shorter allele in the second tumor (reverse loss, indicative of dual clonality). **i** *MGMT* promoter hypermethylation analysis (digested sample), both tumors were not hypermethylated for *MGMT* (*MGMT* digested/*MGMT* undigested ratio of $MGMT_2$ probe <0.30)

In this study 90% of the G:C>A:T transition mutations were located at CpG sites. However, we neither found a significant correlation between *MGMT* hypermethylation and *TP53* mutation, nor a correlation between *MGMT* hypermethylation and specific *TP53* G:C>A:T transition mutations. Zawlik et al. found a significantly higher frequency of *TP53* G:C>A:T transition mutations in *MGMT* promoter hypermethylated glioblastomas, whereas the total frequency of *TP53* mutations in *MGMT* promoter hypermethylated and unmethylated glioblastomas was similar. Interestingly, no association of *MGMT* promoter hypermethylation and the accumulation of G:C>A:T transition mutations in *PTEN* or other genes was found in that study [26], suggesting specific involvement of the *TP53* gene. However, in colorectal tumorigenesis and gastric cancer inactivation of *MGMT* by promoter hypermethylation seemed to be associated with G>A mutations in *K-ras* [39–41]. In accordance with our findings, Jesien-Lewandowicz et al. recently reported no correlation between methylation of the *MGMT* promoter and G:C>A:T *TP53* mutations in a series of 32 primary glioblastomas treated with radiotherapy and surgery [42]. Future studies may reveal whether the specific methylation of *MGMT* is representative of a generalized status of genomic methylation and whether there is a gene-specificity of the G:C>A:T transition mutations.

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