Longitudinal Assessment of Genetic and Epigenetic Markers in Oligodendrogliomas

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Abstract

Purpose: Because little is known about the evolution of genetic and epigenetic changes that occur during tumor progression in oligodendrogliomas, we evaluated these changes in paired early and progressive oligodendrogliomas.

Experimental Design: 1p36, 19q13, 10q22-26, and O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status were assessed in 46 paired early and progressive oligodendrogliomas from 23 patients.

Results: In early tumors, 60.8% were of low grade compared with only 17% low-grade tumors at recurrence. Of 17 early tumors described as pure oligodendrogliomas, 76.5% remained in this lineage, regardless of their grade, whereas others changed to astrocytic tumors. Oligoastrocytic tumors had a significantly higher tendency to transform to astrocytic tumors. All pure oligodendrogliomas with 1p/19q codeletions remained phenotypically unchanged, unlike mixed tumors with codeletions, of which 83% changed their cell lineage. Of tumors with early 1p deletion, 80% remained oligodendroglial at progression, whereas 75% of tumors with an intact 1p changed to astrocytic phenotype. 10q loss was uncommon in both early and progressive tumors. The proportional gain in methylation at progression was 31% for tumors with early 1p deletion, unlike tumors with an intact 1p, which had an 87.5% gain of methylation at progression.

Conclusions: Pure oligodendrogliomas with 1p/19q deletion tend to retain their cell phenotype and genetic profile unlike tumors with no deletions or mixed histology. MGMT promoter methylation is more pronounced at tumor progression, particularly in tumors with an intact 1p. These observations suggest that MGMT promoter methylation is a late event in progressive oligodendrogliomas, and therefore, their chemosensitivity is not necessarily related to MGMT methylation status.

Oligodendrogliomas constitute ~5% to 10% of all glial tumors. A high proportion of oligodendrogliomas contain the combined loss of chromosomes 1p and 19q, which is an uncommon signature in other gliomas and particularly in astrocytomas (1, 2). Genetic subgroups have been identified within the oligodendroglioma phenotype, and those genetic alterations associated with allelic loss on chromosomes 1p and 19q are recognized as favorable markers for predicting response to treatment (3, 4). These allelic losses signify improved outcome, regardless of treatment modality, as verified recently in large prospective randomized clinical trials that evaluated radiation therapy with or without chemotherapy in anaplastic oligodendrogliomas (5, 6).

Another genetic marker, loss of chromosome 10, is one of the most frequent genetic alterations found in gliomas and particularly in astrocytomas. However, it is less frequent in oligodendroglial neoplasms compared with astrocytic tumors (7, 8). Loss of all or most of the long arm of chromosome 10 has been associated with an aggressive tumor behavior and poor prognosis in oligodendrogliomas regardless of the chemosensitivity of these tumors (8). This observation suggests similarities between anaplastic oligodendrogliomas with 10q deletion and malignant astrocytomas containing the same loss. Despite the recognition of a characteristic molecular signature and typical genetic alterations, the pathogenesis of oligodendroglial tumors remains poorly understood, and no oligodendroglial-related genes have yet been identified.

Recent studies have provided evidence that epigenetic gene silencing is a mechanism associated with loss of gene expression that may give tumor cells a growth advantage (9). Hypermethylation of CpG islands in the gene promoters has been associated with reduced expression of various genes including DNA repair genes. O^6-Methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that removes the mutagenic and cytotoxic O^6-alkylguanine lesions induced by either environmental carcinogens or by chemotherapeutic agents. Therefore, high expression of MGMT induces resistance to DNA-alkylating drugs. Recently, MGMT gene transcriptional

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silencing via hypermethylation of the gene promoter correlated with improved survival of glioblastoma multiforme after combined treatment with radiotherapy and temozolomide (10). On the other hand, the presence of MGMT promoter methylation was found to be an independent predictor for an unfavorable clinical course in low-grade diffuse astrocytoma (11).

The status of MGMT promoter hypermethylation and its relevance to tumor progression in oligodendroglial tumors is not well characterized. It was shown that hypermethylation and low MGMT expression may occur frequently in these tumors (12). It was also suggested that it occurs concurrently with hypermethylation of multiple genes and has an association with tumor grade (13). Epigenetic gene silencing in cancer is a mechanism that "addicts" cancer cells to altered signal-transduction pathways during the early stages of tumor development (9). These results imply that promoter methylation is probably an early event in tumor genesis. Little is known about the evolution of genetic and epigenetic alterations that take place during tumor development and progression in oligodendroglial tumors. To further investigate these molecular events, we did a longitudinal assessment of genetic markers (1p, 19q, and 10q) and of epigenetic aberrant MGMT promoter methylation, and correlated them with tumor phenotype in a series of progressive oligodendroglial tumors.

Materials and Methods

Patients and tumors

Forty-six oligodendroglial tumors from 23 patients were obtained at an early phase of the disease process and again at tumor progression (Table 1). The first tumor samples (tumor I) included 15 primary tumors and 8 recurrent tumors. All tumors that were sampled again at a later phase (tumor II) represent progressive tumors. Tumor progression was defined by magnetic resonance imaging and was based on measurable changes in tumor dimensions. To identify a progressive disease, it was required that at least a 25% increase in the largest perpendicular diameter of the cross-section of the enhancing or nonenhancing tumor be present or that a new enhancing mass be

Table 1. Clinical characteristics of the patients, tumor status, and tumor phenotype at initial evaluation (tumor I) and at tumor progression (tumor II)

Patient no.	Gender	Age at diagnosis of primary tumor (y)	Tumor I		Therapy between tumor I and II	Interval between surgical procedures (mo)	Tumor II	Follow-up from diagnosis of primary tumor (mo)	Outcome
			Status (previous therapy)	Histology			Histology		
1	M	65	Primary tumor	AO	TMZ × 3	3	GBM	17	DOD
2	F	51	First progression of LGO (surgery)	AO	TMZ × 11	53	AO	69	A
3	F	17	First progression of LGO (surgery)	O	TMZ × 10	34	AO	128	DOD
4	M	35	First progression of AO (RT, PCV × 3)	AO	TMZ × 15	17	GBM	43	DOD
5	M	26	Primary tumor	OA	None	37	Astro	37	A
6	F	51	Primary tumor	AO	TMZ × 9, RT	23	GBM	29	DOD
7	M	48	First progression of AO (PCV × 2, RT)	AO	TMZ × 14	24	AO	78	DOD
8	F	24	Primary tumor	AOA	TMZ × 14, RT	19	GBM	31	DOD
9	M	31	First progression of LGO (surgery)	O	PCV × 4, TMZ × 14	61	AO	79	A
10	F	70	Primary tumor	AO	TMZ × 6, RT	16	AO	21	DOD
11	M	41	Primary tumor	O	PCV × 6, TMZ × 8	33	AO	64	A
12	M	40	First progression of LGO (surgery, RT)	O	TMZ × 13	20	AO	152	A
13	M	33	Primary tumor	OA	None	15	AO	37	A
14	F	30	Primary tumor	OA	PCV × 2, TMZ × 20, RT	77	GBM	84	DOD
15	M	31	Primary tumor	O	None	24	O	88	DOD
16	M	47	Primary tumor	O	TMZ × 24	64	AO	102	A
17	F	18	Primary tumor	O	None	21	AO	58	DOD
18	F	36	Primary tumor	O	None	13	AO	35	A
19	M	44	Second progression of LGO (surgery, RT)	AO	PCV × 3	14	AO	124	A
20	M	34	Primary tumor	OA	None	55	OA	78	A
21	M	36	First progression of LGO (RT)	O	PCV × 4, TMZ × 15	77	AO	129	DOD
22	M	30	Primary tumor	O	TMZ × 24	40	O	57	A
23	M	48	Primary tumor	AOA	PCV × 6, RT	27	GBM	79	A

Abbreviations: LGO, low-grade oligodendrogliomas; Astro, astrocytoma (WHO II); AO, anaplastic oligodendroglioma (WHO III); AOA, anaplastic oligoastrocytoma (WHO III); O, oligodendroglioma (WHO II); OA, oligoastrocytoma (WHO II); GBM, glioblastoma multiforme (WHO IV); RT, radiotherapy; TMZ, temozolomide; PCV, procarbazine-CCNU-vincristine; DOD, dead of disease; A, alive.

Table 2. Primer pairs of the microsatellite loci on chromosomes 1p, 19q, and 10q

Chromosome	Locus	Forward primer sequence	Reverse primer sequence
1p	*D1S199*	GGTGACAGAGTGAGACCCTG	CAAAGACCATGTGCTCCGTA
	D1S226	GCTAGTCAGGCATGAGCG	GGTCACTTGACATTCGTGG
	D1S186	TAGCTCATCCCCCCCTTTCT	CCCCTCCTTCCTGCCGCT
	D1S312	CAGCCTTCCCCACAACTTTA	TTCCAAACAGCAGGGGAG
10q	*D10S185*	TCCTATGCTTTCATTTGCCCA	CAAGACACACGATGTGCCAG
	D10S1765	ACACTTACATAGTGCTTTCTGCG	CAGCCTCCCAAAGTTGC
19q	*D19S112*	GCCAGCCATTCAGTCATTTGAAG	CTGAAAGACACGTCACACTGGT
	D19S918	AAAGGCTTGATTACCCCCGA	GATTACAGGCGTGAGCACCG

recognized. Patients were referred for repeat tumor resection (tumor II samples) as part of their treatment plan when tumor progression was identified. When a biopsy rather than surgery was done at progression, it was usually aimed at differentiating a change in tumor grade or histology from delayed radiation necrosis in those previously treated by radiation therapy. One pathologist (D. Soffer) assessed all tumors for their histologic diagnosis both at initial evaluation and again at tumor progression.

DNA extraction and microsatellite analysis

All patients signed an informed consent form for DNA extraction and analysis. The DNA was extracted from tumor and blood samples. DNA was extracted from leukocytes and serum at 200 μL aliquots by a Blood-DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA from paraffin-embedded tumor tissue was isolated by the Paraffix kit (Syntezza Bioscience, Jerusalem, Israel) according to the manufacturer's instructions.

Microsatellite analysis. For the PCR-based loss of heterozygosity analysis, eight primer pairs of the microsatellite loci labeled with one of three fluorochromes, FAM, HEX, or NED, were obtained from Applied Biosystems (Foster City, CA) on chromosomes 1p, 19q, and 10q (Table 2).

Loss of heterozygosity was determined by comparing paired tumor DNA samples with matched normal leukocyte DNA. PCR was done on each patient's samples (normal lymphocyte DNA and tumor DNA) in a final volume of 25 μL containing two primer pairs (3 pmol of each primer), 25 ng of DNA, 10 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 0.2 mmol/L of deoxyribonucleoside triphosphates, 2.5 mmol/L of $MgCl_2$ and 0.6 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR cycling conditions were 95°C for 9 min once, 42 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s. Amplified PCR products were electrophoresed in denaturing 5% polyacrylamide gels on an ABI Prism 310 automated DNA sequencer (Applied Biosystems). Analysis of the collected data was done with GeneScan Analysis software version 3.1 (Applied Biosystems). Loss of heterozygosity was inferred by a 70% reduction of allele signal intensity in tumor samples relative to that of the matched constitutional DNA samples.

Analysis of MGMT promoter methylation status

The methylation status of the MGMT promoter was determined on tumor DNA. Genomic DNA (500 ng) from each sample was chemically modified by sodium bisulfite to convert all unmethylated cytosines to uracils, although leaving methylcytosines unaltered (EZ DNA methylation kit; Zymo Research, Oregon, CA). Aliquots of 2 to 4 μL of the converted DNA were subjected to methylation-specific PCR using two primer sets designed for amplifying the methylated or unmethylated allele of the MGMT promotor. The primer sequences of MGMT for the unmethylated reaction was: 5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ (forward primer) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (reverse primer); and for the methylated reaction: 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ (forward primer) and 5′-GCACTCTTCCGAAAACGAA ACG-3′ (reverse primer). PCR was done under the following conditions: an initial melting step of 10 min at 95°C; followed by 50 cycles of 20 s at 95°C, 20 s at 59°C and 45 s at 72°C; and a final elongation step of 4 min at 72°C in Gene Amp 9700 thermocycler (Applied Biosystems) using AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplified products were separated on 3.5% methaphore gel and visualized under UV illumination.

Immunohistochemistry staining for MGMT

Five-micrometer-thick sections were deparaffinized in xylene, dehydrated, and then incubated with 10 mmol/L of sodium citrate buffer (pH 6.5) and heated by microwave (500 W) for 30 min. The samples were left in the heated buffer for 10 min at room temperature. Following 5 min of treatment in 3% hydrogen peroxide, blocking was achieved by incubation with 3% bovine serum albumin for 30 min followed by 1 h incubation with mouse monoclonal antibodies against human MGMT (1:50 dilution, MAB16200; Chemicon, Temecula, CA) at 37°C. Then, sections were treated with secondary antibody (biotinylated anti-mouse ABC Elite kit; Vector Laboratories, Burlingame, CA) for 30 min at room temperature, and followed by avidin-biotin complex for 20 min and developed with diaminobenzidine substrate (Sigma, Rehovot, Israel) according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin (Sigma). Negative controls were carried out by omission of the primary antibody. The level of MGMT protein expression was defined semiquantitatively according to the fraction of positive nuclear staining and was scored as high (50-100% positive nuclear staining), intermediate (10-49%), or low (<10% nuclear staining). The semiquantitative evaluation was done by one pathologist (Y. Fellig) blinded to all patient's details.

Statistical Analysis

To compare the means between two different groups, a *t* test was used. This test compared the interval between surgical procedures in low-grade and high-grade tumors, and rate of methylation of tumor I in neoplasms with or without 1p loss and gain of methylation by 1p status. The paired samples *t* test compared means between two different progress points and was used to evaluate changes in tumor grade, as well as changes in promoter methylation between tumor I and tumor II. All reported *t* test results were two-sided. Cross-tabulation with χ^2 dependency test was used for testing the dependency of two nominal variables. It was used to detect the dependency between phenotypes of tumor I and tumor II, 1p status of phenotypes of tumor I and tumor II, phenotypes of tumor I and tumor II in the case of 1p/19q codeletion, and dependency between 1p status and MGMT methylation. One-way ANOVA with simultaneous average comparisons was used to study the dependency between 1p status, MGMT methylation, and MGMT expression. Linear regression analysis was used to search for a multiparameter model that would explain dependent variables related to gain of methylation in recurrent tumors. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics and tumor phenotype. The clinical characteristics of the patients and tumor phenotypes at initial evaluation and at tumor progression are described in Table 1.

There were 15 males and 8 females, ranging in age between 17 and 70 years (median, 36 years). Tumor histology at initial evaluation included 14 WHO grade 2 tumors (10 oligodendrogliomas and 4 oligoastrocytomas), and 9 WHO grade 3 tumors (7 anaplastic oligodendrogliomas and 2 anaplastic oligoastrocytomas). Eight patients' tumors were first analyzed at progression, as they presented at our center with a progressive disease and no paraffin blocks of the primary tumor were available for genetic studies. Six of these eight tumors were previously diagnosed as low-grade oligodendrogliomas (WHO II) and four of them retained that diagnosis at the time of evaluation of tumor I, whereas two were now diagnosed as anaplastic tumors (WHO III). Two other tumors known to be anaplastic remained in this category at first analysis.

All tumor samples were obtained by surgical procedures classified as either an open craniotomy or as a stereotactic tumor biopsy. A total of 32 craniotomies and 14 biopsies were done and they were equally divided between tumor I and tumor II groups. The interval between the two surgical procedures that collected tumor samples ranged between 3 and 77 months. For tumors that were anaplastic at initial evaluation (tumor I), the median interval was 19 months (range, 3-53) and it was significantly shorter than the median interval of 35.5 months (range, 13-77) of low-grade tumors at first evaluation (t test, $P = 0.03$).

At initial evaluation, 14 of the 23 (60.8%) tumors were of low grade (WHO II). A clear trend toward increase in tumor grade was observed. The histology of the 23 progressive tumors (tumor II) revealed that only 4 tumors (17%) were still of low grade whereas the remaining 19 tumors (82.6%) were now diagnosed as high-grade neoplasms (WHO III; paired t test, $P = 0.0000$).

Tumor phenotype regardless of its grade was assessed on first evaluation as pure oligodendroglial in 17 cases (73.9%) and as oligoastroglial in the other 6 (26%). At progression, 14 of the 17 (82.3%) pure oligodendroglial tumors remained in this lineage notwithstanding an increase in tumor grade that was observed in 8 of the 14 tumors (57%). Only 3 of the 17 pure oligodendroglial tumors (17.6%) changed to primarily astrocytic tumors; all were grade 3 tumors that transformed to glioblastoma multiforme (patient nos. 1, 4, and 6). Of the six oligoastroglial tumors, four (66.6%) transformed to astrocytic tumors at progression (patient nos. 5, 8, 14, and 23), one remained unchanged (patient no. 20), and one oligoastrocytoma (WHO II) was predominantly anaplastic oligodendroglioma at repeat evaluation (patient no. 13). To sum up, oligoastrocytic tumors were more likely to present as predominantly astrocytic phenotype at progression and differed significantly from pure oligodendroglial neoplasms in that aspect ($\chi^2 = 7.812$, $P = 0.02$).

Molecular genetics and tumor phenotype. The results of evaluation of allelic loss on chromosomes 1p, 19q, and 10q for each sample of tumor I and tumor II groups are described in Table 3. At initial assessment, there were eight tumors with

Table 3. Tumor phenotype, chromosomal allelic status and MGMT promoter methylation status on early evaluation (tumor I) and at tumor progression (tumor II)

Patient no.	Phenotype I	Tumor I				Phenotype II	Tumor II			
		Allelic status			MGMT methylation		Allelic status			MGMT methylation
		1p	19q	10q			1p	19q	10q	
1	AO	+	–	ND	U	GBM	+	+	–	M
2	AO	+	–	ND	U	AO	+	+	+	M
3	O	+	+	+	U	AO	+	+	+	M
4	AO	+	–	+	U	GBM	+	–	+	M
5	OA	+	–	+	U	A	+	–	+	M
6	AO	+	+	–	U	GBM	+	+	–	M
7	AO	+	+	+	U	AO	+	+	+	M
8	AOA	+	–	+	U	GBM	+	+	+	U
9	O	–	–	+	M	AO	–	–	+	M
10	AO	–	–	ND	M	AO	–	–	ND	M
11	O	–	–	+	M	AO	–	–	+	M
12	O	–	–	+	U	AO	–	–	+	M
13	OA	–	–	+	U	AO	+	+	+	M
14	OA	–	–	+	U	GBM	+	+	+	M
15	O	–	–	ND	U	O	–*	–	+	M
16	O	–	–	ND	U	AO	–	–	+	U
17	O	–	H	–	U	AO	–	H	+	U
18	O	–	–	H	U	AO	–	–	H	U
19	AO	–	–	+	U	AO	–	–	+	U
20	OA	–	–	–	U	OA	+	+	+	U
21	O	–	–	+	ND	AO	–	–	+	U
22	O	–	–	+	ND	O	–	–	+	ND
23	AOA	–	–	+	M	GBM	+	+	–	M

Abbreviations: A, astrocytoma; AO, anaplastic oligodendrogliomas; AOA, anaplastic oligoastrocytoma; O, oligodendrogliomas; OA, oligoastrocytoma; GBM, glioblastoma multiforme; H, homozygosis; (+), intact allelic status; (–), allelic loss; M, methylated MGMT promoter; ND, not determined; U, unmethylated MGMT promoter.
*Allelic loss was detected on different alleles in tumor I and tumor II.

no loss of 1p arm (+1p) and 15 (65%) with 1p deletion (−1p). All the tumors with 1p deletion in which 19q could be assessed contained a 19q codeletion. Five of the tumors with an intact 1p had 19q loss and only three tumors presented no loss on these chromosomes. The median interval between the two surgical procedures, when evaluated according to 1p status, did not differ significantly. It was 27 months (range, 14-77) for tumors with 1p deletion and 23.5 months (range, 3-53) for tumors with an intact 1p.

A change in the molecular signature of either chromosomes 1p or 19q was observed in 8 (34.7%) of the 23 samples of tumor II (Table 3; Fig. 1). In seven tumors, a deletion that was previously detected in tumor I was no longer seen in tumor II, probably suggesting that a different dominant clone was now over-represented. It should be noted that of the 15 tumors with an early signature of 1p/19q codeletion, the only ones that exhibited a genotypic change at progression (no deletions) were those in which the initial phenotype was oligoastroglial (patient nos. 13, 14, 20, and 23).

Of particular interest is the unique change observed in patient no. 15 (Fig. 1). The primary tumor histology in this case was of oligodendroglioma (WHO II) with codeletion of 1p/19q. Twenty-four months later, the biopsy of the progressive tumor showed that the histology was still of an oligodendroglioma with an overall similar loss of heterozygosity status that contained 1p/19q codeletion. However, the microsatellite analysis of 1p showed that the chromosomal loss observed in the primary tumor and the one detected at recurrence involved different alleles. This is probably another indication that a different clonal expansion is overexpressed in the progressive tumor.

The relationships between 1p status of tumor I and tumor phenotypes at both initial evaluation and at recurrence were assessed. Of the 15 tumors with an early 1p deletion, 12 (80%) maintained oligodendroglial phenotype at progression regardless of tumor grade, whereas 5 (62.5%) of the 8 tumors with an intact 1p on early assessment presented a phenotypic change to a predominantly astrocytic neoplasm at progression. It was found that 1p status of tumor I had a significant influence on tumor phenotype at progression (χ^2 = 8.83, P = 0.01). The oligodendroglial phenotype of all 11 tumors that were initially characterized as pure oligodendroglial tumors remained unchanged at progression, regardless of tumor grade, once they contained codeletion of 1p/19q. Although of the four oligoastrocytic tumors with codeletion, only one presented an oligodendroglial phenotype at progression. The phenotype of the early tumor (whether pure or mixed tumor) had a significant influence on the cell type histology at progression despite the presence of a similar genetic profile indicating 1p/19q codeletion (χ^2 = 10.31, P = 0.006).

Loss of 10q was uncommon in both tumor I and tumor II samples. Allelic loss of 10q was found in 3 (16.6%) of the 18 tumors evaluated at the early phase (patient nos. 6, 17, and 20), and in 3 (13.6%) of 22 samples at tumor progression (patient nos. 1, 6, and 23). In two tumors that contained deletions in early samples, no loss was found at tumor progression (patient nos. 17 and 20), and one tumor acquired a deletion at progression (patient no. 23). Thirteen (76%) of the 17 tumors that had an intact 10q at initial evaluation remained unchanged at tumor progression.

MGMT promoter methylation and tumor genotype. MGMT promoter methylation was detected in 4 (19%) of the 21 early tumors. A significant increase in MGMT promoter methylation was observed in the progressive tumors of which 15 (68%) of the 22 tumors had MGMT methylation (paired t test, P = 0.000). MGMT was uniformly unmethylated in early tumors with an intact 1p, whereas four (30.7%) of the tumors with a 1p deletion contained methylated MGMT at initial evaluation (t test, P = 0.04). All tumors that presented with MGMT methylation contained methylation in the progressive tumor as well. Figure 2 shows examples of two tumors with an unchanged MGMT methylation status and of one tumor that acquired promoter methylation at progression. We compared the proportional gain in methylation observed at tumor progression for tumors with initial 1p loss and for those with an early intact 1p. The proportional gain in methylation at tumor progression (expressed as no gain = 0, gain = 1) was only 30.7% for tumors initially characterized by 1p deletion. It was significantly lower than the proportional gain observed in tumors with an intact 1p, which had an 87.5% acquisition of methylation at recurrence (t test, P = 0.006). Multiple variables were entered into linear regression ANOVA in order to find a multiparameter model that may indicate association with methylation acquisition at tumor progression. The variables included age at diagnosis of primary tumor; age at tumor I biopsy; interval between the two surgical procedures; tumor phenotype and tumor grade, defined as either pure oligodendroglial or oligoastroglial at initial evaluation; 1p, 19q, and 10q status of tumor I; duration of follow-up since diagnosis of primary tumor; and tumor status—whether primary tumor or a progressive one at first evaluation. This analysis did not detect any model with significant dependent variables associated with

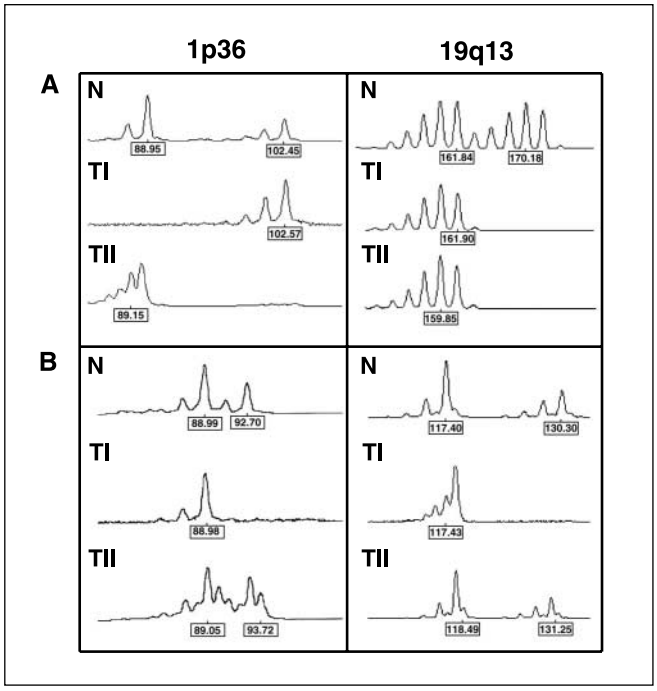

Fig. 1. Capillary electropherograms of PCR products resulting from amplification of microsatellite loci on chromosomes 1p36 and 19q13. Normal alleles (*N*) amplified from blood DNA are compared with the alleles in the early tumor (*TI*) and recurrent (*TII*) neoplasm. *A,* tumor samples from patient no. 15 contained 1p/19q codeletion but the deletions in 1p involved different alleles. The 19q deletions remained unchanged. *B,* tumor samples from patient no. 23 revealed that TI contained 1p/19q codeletion, whereas the recurrent tumor showed intact 1p/19q chromosomes.

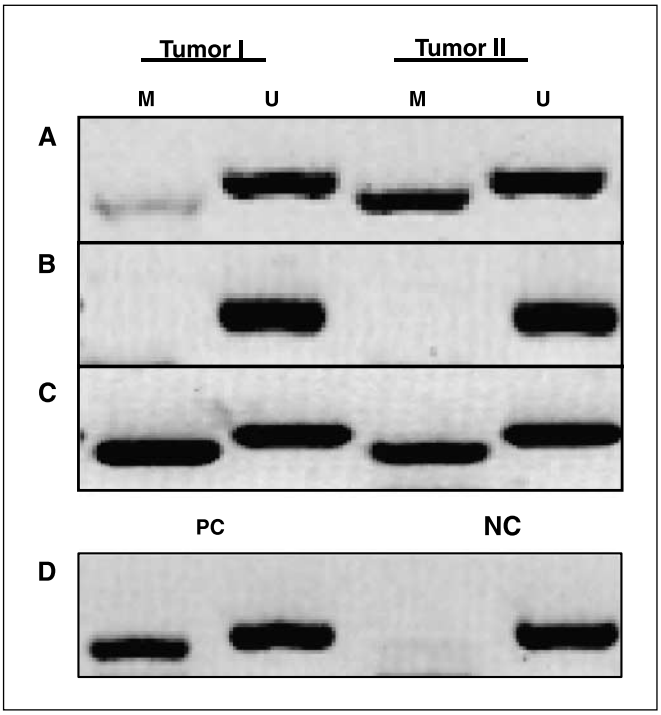

Fig. 2. Methylation-specific PCR analyses of MGMT promoter. PCR was preformed on the DNA extracted from early (*Tumor I*) and recurrent (*Tumor II*) tumor samples, using unmethylated (*U*) and methylated (*M*) specific primers. *A,* early tumor of patient no. 14 showed no methylation of MGMT promoter whereas the recurrent tumor contained methylated promoter. *B,* both early and recurrent tumors of patient no. 16 show no methylation of MGMT promoter. *C,* both early and recurrent tumors of patient no. 9 contained methylated MGMT promoter. *D,* positive control for methylated DNA (*PC*); normal control (DNA from a normal blood sample) with an unmethylated MGMT (*NC*).

gain of methylation at tumor progression. We carefully examined the possibility that the eight tumors that were first evaluated at early progression (tumor I; patient nos. 2, 3, 4, 7, 9, 12, 19, and 21) may have manifested a different pattern of methylation because they were not the primary tumors. Only one of them (14%) had a methylated MGMT promoter on initial evaluation (patient no. 9) and all others had an unmethylated promoter similar to the findings in primary tumors. At further progression (tumor II), these tumors showed the observed trend of acquisition of methylation, and five tumors gained promoter methylation. Overall, these tumors manifested similar evolution as the true primary tumors of tumor I group.

MGMT protein expression. MGMT protein expression was assessed by immunohistochemistry staining in 13 tumors. It was done whenever good-quality unstained paraffin sections were available for immunohistochemical study. The results of the semiquantitative evaluation are presented in Table 4. We analyzed the results of the percentage of positive nuclear staining in relation to the promoter methylation status and to 1p status.

Tumors with an intact 1p had a significantly higher mean MGMT expression (mean ± SE, 62 ± 13.6%) when compared with those with 1p deletion (9.8 ± 3.0%; *t* test, $P = 0.01$). The dependence between 1p status and MGMT expression was statistically significant ($\chi^2 = 9.24$, $P = 0.002$) whereas no significant dependency was found between MGMT methylation status and MGMT expression. One-way ANOVA with simultaneous average comparisons indicates that 1p status rather than MGMT methylation is related to the significant difference observed in MGMT expression ($F = 8.49$, $P = 0.008$).

Discussion

In this study, we did a longitudinal assessment of oligodendroglial tumors in order to detect changes in both tumor phenotype and genotype. In addition, we evaluated deviations in the status of MGMT gene promoter methylation that are induced by epigenetic alterations. Longitudinal assessment of oligodendroglial tumors was included in two previous studies (14, 15) that evaluated chromosomal allelic losses in a large number of oligodendroglial tumors. These series contained a limited number of primary-recurrent pairs of neoplasms. However, no previous publication assessed changes in the methylation status of MGMT promoter along the course of tumor progression and evolution.

Table 4. MGMT promoter methylation and protein expression assessed semiquantitatively by immunohistochemistry staining

Patient no.	Phenotype (tumor no.)	1p status	MGMT methylation	MGMT expression (% nuclear staining)
2	AO (I)	+	U	60
3	O (I)	+	U	75
4	AO (I)	+	U	85
7	AO (II)	+	U	10
8	GBM (II)	+	U	80
9	O (I)	–	M	10
11	O (I)	–	M	6
11	AO (II)	–	M	6
12	O (I)	–	U	2
20	OA (I)	–	U	5
20	OA (II)	–	U	30
21	O (I)	–	ND	10
21	AO (II)	–	U	10

Abbreviations: AO, anaplastic oligodendroglioma; O, oligodendroglioma; OA, oligoastrocytoma; GBM, glioblastoma multiforme; (+), intact allelic status; (–), allelic loss; M, methylated MGMT promoter; U, unmethylated MGMT promoter; ND, not determined.

Tumor phenotype and genotype. Our findings suggest that two distinct subsets of oligodendroglial tumors could be identified based on early evaluation of both tumor phenotype and genotype. Pure oligodendroglial tumors that contain early codeletion of 1p/19q retain both their oligodendroglial phenotype and their original genotype at progression, regardless of changes in tumor grade. On the other hand, those tumors in which early genotype contains an intact 1p, or alternatively, their initial phenotype is oligoastroglial, tend to manifest at progression a predominant astrocytic lineage. These predominantly astrocytic tumors manifest the genetic hallmark of an intact 1p at progression, even if the tumor initially presented with 1p/19q codeletion. This indicates that pure oligodendroglial neoplasms are homogeneous in their genotype and cell lineage, as can be expected when a monoclonal origin is assumed. Several previous studies reported similar observations and support the hypothesis that oligodendroglial tumors arise monoclonally from a single precursor cell (1, 7, 14, 16). On the other hand, oligoastrocytomas are tumors that contain admixed neoplastic cell types but may still show 1p/19q alterations that are genetically related to oligodendrogliomas (1, 14, 17). It was previously suggested that oligoastrocytomas with 1p/19q loss are histologically oligodendroglioma-predominant tumors (1). Their tendency to present at progression with astrocytic-predominant phenotype in our cohort is not surprising because clonal expansion of the astrocytic component probably took place. This assumption is also supported by the observed change in tumor genotype from codeletion of 1p/19q to an intact chromosomal profile in the progressive tumor. An interesting study previously reported that in oligoastrocytic tumors, unlike in oligodendroglial tumors, loss of 1p/19q did not correlate with survival, probably due to the negative prognostic effect of the astrocytic elements of the tumor (18). Similarly, tumors that presented no 1p loss at diagnosis have a worse prognosis when compared with their counterparts that have codeletions (4–6, 19). These studies may suggest that tumors with no 1p loss are genetically related to astrocytomas, and our observation that they have a significant increased tendency for a predominant astrocytic manifestation at tumor progression provides further support for this. Notwithstanding, it is possible that limited sampling of the tumors may have lead us to inaccurate conclusions, and therefore, the above suggestions should be further verified in the future.

There are two previous studies that have done a longitudinal assessment of the molecular pathology of oligodendroglial tumors (7, 14). Similar to our findings, both studies showed that ~70% of their cases retained the initial histologic differentiation in subsequent samples, whereas the plasticity of the phenotype was evident in the others. Acquisition of genetic change was seen in 29% of 31 tumors (7), which is also comparable to our observation of 39% alterations once 1p/19q and 10q are both taken into account.

Loss of chromosome 10 was an infrequent event in the current study, both on initial evaluation and also at tumor progression. Similar observations were noted in previous reports, in which 10q loss ranged between 1% and 27% (7, 8, 14). It was even suggested that 10q deletion was sufficiently rare in pure oligodendrogliomas to suggest the possibility of an alternate diagnosis such as small cell glioblastoma (14). Interestingly, all three progressive tumors that manifested 10q loss in our small cohort were morphologically diagnosed at that time as glioblastoma multiforme. The observation that 10q deletion and 1p loss are inversely related in anaplastic oligodendrogliomas and that tumors with 10q loss were associated with poor chemosensitivity suggested similarities between anaplastic oligodendrogliomas with 10q loss and malignant astrocytomas with the same deletion (8). This sustains the distinction between two subsets of oligodendrogliomas as delineated by our longitudinal analysis and by previous reports. The findings that pure oligodendrogliomas with 1p/19q codeletion tend to maintain their cell lineage and genotype in the current study and in previous reports (1, 7, 14, 20) supports the hypothesis that 1p/19q codeletion represents an early genetic event in the biologically more favorable subset of these neoplasms.

MGMT promoter methylation and protein expression. MGMT is ubiquitously expressed in normal human tissues and is often overexpressed in all types of human tumors. The expression of MGMT in tumors has a protective effect against cell death induced by alkylating agents (10, 21, 22). However, MGMT is silenced in various cancers and cancer cell lines, and it is believed that the silencing is associated with DNA methylation at a discrete region of the promoter CpG island (21–23). Low expression of MGMT has been shown in various studies that evaluated low- and high-grade oligodendrogliomas (12, 24–27), and it has been suggested that it may be associated with the chemosensitivity of these tumors. However, no previous study has done a longitudinal evaluation of MGMT promoter methylation and compared the methylation status of the early tumor to that of the progressive disease. This type of assessment done in the current study shows that acquisition of aberrant methylation of MGMT promoter is allied with tumor progression and that it is not necessarily an indicator of a favorable subtype of these tumors. Yet, it should be noted that the majority of our patients received chemotherapy prior to tumor progression and it can be argued that it could have provided selective pressures for proliferation of the more resistant tumor clones that accumulate aberrant genetic alterations.

We found that early tumors had a 19% rate of MGMT promoter methylation, but at progression, aberrant methylation became frequent, being detected in 68% of recurrent tumors. This observation was congruent with recent recognition that MGMT hypermethylation alone is not a good prognostic factor per se. In fact, it signifies poor prognosis, probably owing to the fact that tumors with epigenetic silencing of MGMT accumulate more mutations (22). Accordingly, accumulation of MGMT methylation in systemic tumors is associated with an increasing tumor stage and with CpG island methylation of other genes (21–23, 28). Similar evidence suggests that in oligodendrogliomas, hypermethylation of CpG islands is not limited to single genes but occurs simultaneously in multiple genes located at different chromosomes (13). Concordant hypermethylation of several genes is predominantly found in anaplastic oligodendrogliomas, suggesting that more genes become susceptible to hypermethylation during a later stage of tumor development.

With the perception that acquisition of MGMT methylation is associated with increasing tumor grade and probably grim prognosis, we searched our findings for a possible disparity between the two genetic subtypes of oligodendrogliomas stratified according to 1p status. We showed that at tumor

progression, the proportional gain of methylation differed significantly between the two subtypes, with a higher methylation gain observed in tumors with an intact 1p. This observation fits the concept that gene silencing by promoter hypermethylation is, in general, a poor prognostic factor, and it occurs more frequently in tumors with an intact 1p that have worse prognosis (21–23, 28, 29).

As opposed to the above perception, several studies have shown that MGMT promoter methylation and transcriptional gene silencing are associated in glial tumors with either the molecular signature that implicates tumor chemosensitivity (1p/19q deletions) or with chemoresponsiveness and improved survival (4–6, 10, 12, 19, 24–27). In most cases, a low level of MGMT is a good predictive marker for chemoresponsiveness to alkylating agents and, accordingly, several studies showed that oligodendroglial tumors show either reduced mRNA levels or low MGMT expression on immunohistochemistry evaluation (12, 24, 26, 27). However, the correlation between MGMT promoter hypermethylation and MGMT protein expression is not always apparent in glial tumors (12, 29, 30), as is the case in our current findings done on analysis of a limited study cohort. The inconsistency between MGMT promoter hypermethylation and protein expression in gliomas is similar to the observations in other neoplasms (31, 32) and to the fact that these findings are not limited to the MGMT gene (33–35). It indicates that the regulation of gene expression, and particularly of MGMT expression, is a complex process in which abnormal promoter methylation is only one of various formative factors. It should be noted that several studies indicate that grade of methylation, both in the promoter region and in neighboring sequences, may regulate gene expression (30, 34, 36, 37). The methylation-specific PCR method is a highly sensitive qualitative technique that identifies the methylation of specific CpG sites within the promoter but is unable to detect differences in the grade of methylation in other CpG sites or neighboring sequences that are sometimes crucial for the silencing of gene expression. As a result, methylation-specific PCR identification of promoter methylation should not be automatically interpreted as associated with loss of protein expression.

Our limited results in the current study indicate that 1p status rather than MGMT promoter methylation was associated with MGMT protein expression. This finding needs to be validated in a larger cohort of oligodendroglial tumors. Likewise, we have reported in a previous study that in low-grade oligodendrogliomas, MGMT protein expression correlates with 1p status (24). Deletion of 1p was associated with low MGMT expression, whereas high expression was prominent in tumors with an intact 1p.

Conclusions

The longitudinal assessment in the current study indicates that pure oligodendroglial tumors with 1p/19q deletion tend to retain their cell phenotype and genetic profile at tumor progression, unlike tumors with no deletions or with admixed cell phenotype. MGMT promoter methylation is predominantly pronounced at tumor progression, implying that this is not an early event in the genesis of oligodendroglial tumors. It is probably related to the global accumulation of genetic aberrations associated with tumor progression and does not necessarily signify a good prognosis, as is evident in tumors with an intact 1p. It is suggested that MGMT protein expression is related to 1p status rather than to MGMT promoter methylation, but this requires further verification. That MGMT promoter methylation is the only relevant molecular factor in the tumor's chemosensitivity is probably an oversimplification. It is likely that there are other contributing factors, yet to be discovered.

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