Real-Time Quantitative PCR Analysis of Gene Dosages Reveals Gene Amplification in Low-Grade Oligodendrogliomas

M. Eva Alonso, PhD,[1] M. Josefa Bello, PhD,[1] Dolores Arjona, PhD,[1] Victor Martinez-Glez, MD,[1] Jose M. de Campos, MD, PhD,[2] Alberto Isla, MD, PhD,[3] M. Elena Kusak, MD,[2] Jesús Vaquero, MD, PhD,[4] Manuel Gutierrez, MD, PhD,[5] Jose L. Sarasa, MD, PhD,[6] and Juan A. Rey, PhD[1]

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Abstract

*Proto-oncogene amplification is an important alteration that is present in about 45% to 50% of high-grade human gliomas. We studied this mechanism in 8 genes (cyclin-dependent kinase-4 [*CDK4*],* MDM2*,* MDM4*, renin-angiotensin system-1,* ELF3, GAC1*, human epidermal growth factor receptor-2, and platelet-derived growth factor receptor-A gene) in a series of 40 oligodendrogliomas (World Health Organization (WHO) grade II, 21; WHO grade III, 13; and WHO grade II-III oligoastrocytomas, 6) using real-time quantitative polymerase chain reaction. Amplification of at least 1 of these genes was detected in 58% of samples (23/40). By histopathologic grade, 67% of grade II oligodendrogliomas (14/21), 46% of grade III anaplastic oligodendrogliomas (6/13), and 50% of mixed oligoastrocytomas (3/6) were positive for amplification of at least 1 gene.* CDK4, MDM2, *and* GAC1 *were the most frequently involved genes (12/40 [30%], 12/40 [30%], and 13/40 [33%], respectively). Our findings demonstrate gene amplification in low-grade samples indicating that it is an important alteration in the early steps of oligodendroglioma development and, therefore, might be considered a molecular mechanism leading to malignant progression toward anaplastic forms.*

Oligodendrogliomas are primary tumors of the central nervous system that occur predominantly in the cerebral hemispheres and are more frequent in the fifth and sixth decades of life.[1] They account for 5% to 18% of all gliomas and include pure oligodendrogliomas, corresponding primarily to World Health Organization (WHO) grades II and III (anaplastic) of malignancy, and mixed oligoastrocytomas.[1] Chromosomal, genetic, and epigenetic aberrations in oligodendrogliomas involved with their development and progression have been identified and include allelic losses at 1p and 19q,[2,3] gene mutations of *PTEN* and *p16^{ink4A}*,[4,5] and aberrant promoter methylation of certain tumor-related genes.[6,7] Oncogene amplification is involved in the development of many solid tumors in humans and is a reflection of the genetic instability of tumor cells; proto-oncogene amplification, therefore, has been associated with tumor stage and progression in human gliomas.[8] This genetic alteration induces amplification of different tumor-related genes and leads to altered expression of proteins that have important roles in the regulation of cell proliferation.[8]

Some genes with potential oncogenic features located at 12q13-14 are amplified and their proteins overexpressed in malignant gliomas.[9] These genes are *CDK4* (cyclin-dependent kinase-4 involved in the cell cycle regulation), *SAS* (associated with growth alterations and frequently coamplified with *CDK4*), and *MDM2* (oncogene regulator of *TP53*).[9,10] Because individual malignant gliomas show *CDK4/SAS* amplification but no *MDM2* amplification or vice versa, the possibility exists of a common amplification target gene located between *CDK4* and *MDM2*. These amplification data between *CDK4/SAS* and *MDM2* provide additional evidence that these genes would represent 2 independent targets for amplification in high-grade gliomas.[9-11]

MDM4, also known as *MDMX,* located at 1q32, encodes for a p53-binding protein that shares structural similarities with Mdm2 and has been proposed as a negative regulator of p53 function.[12] Other genes at 1q32 that can be coamplified with *MDM4* in high-grade gliomas are *GAC1* (also overexpressed in these brain tumors), *REN1* (renin-angiotensin system gene implicated in tumoral cell supply), and *ELF3* (a transcription factor with a role in cellular differentiation).[13] The epidermal growth factor receptor (*EGFR*) gene frequently is amplified in oligodendrogliomas and, together with the other 3 members of this gene family, has an important role in the development of other tumor types.[14] For example, amplification of human epidermal growth factor receptor-2 (*HER-2*) is one of the first consistent genetic alterations found in breast cancer, but few studies have so far been performed on *HER-2* amplification in gliomas.[15] Another important growth factor is the platelet-derived growth factor (PDGF); its signaling activation system has been involved in the development and malignant progression of gliomas.[16] Overexpression of PDGF system components, particularly the α subtype receptor (PDGFRA), is common in glial tumors, primarily those with oligodendroglial differentiation and highly anaplastic features.[17]

All of these findings suggest that overexpression of the aforementioned genes might be related to the gene amplification process. Because data are scarce on this subject, we studied a series of 40 oligodendrogliomas by quantitative polymerase chain reaction (PCR) of 8 genes located at 12q13-14 (*CDK4* and *MDM2*), 1q32 (*MDM4, GAC1, REN1,* and *ELF3*), 17q21 (*HER-2*), and 4q11 (*PDGFRA*) to determine the role that their amplification might have in the development of oligodendroglioma.

Materials and Methods

Tissue Samples and DNA Preparation

Tumor biopsy specimens from 40 patients with oligodendroglial tumors were obtained during surgical procedures and frozen immediately at –80ºC until use. All samples were classified by histologic examination and graded according to the WHO guidelines.[1] The 40 tumors consisted of 21 WHO grade II oligodendrogliomas, 13 WHO grade III anaplastic oligodendrogliomas, and 4 WHO grade II and 2 WHO grade III mixed oligoastrocytomas. One case (O-9t1) progressed from an oligodendroglioma to an anaplastic oligodendroglioma (AO-9t2). The tumor cell content in the samples was estimated by histologic examination to be approximately 75% to 80%. This cohort of patients has been studied previously for various genetic abnormalities, including a mutational study of *TP53* gene family members, allelic status at 1p and 19q, and multiple CpG island methylation analysis.[3,7] DNA was prepared from frozen tissue samples and blood samples using standard methods, as described previously.[7]

Quantitative PCR

Quantitative real-time PCR was performed using a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) to analyze the amplification status at *CDK4, MDM2, MDM4, GAC1, REN1, ELF3, HER-2,* and *PDGFRA*. Genomic amplification of gene exons was performed with an amount of starting DNA of 50 nmol/L and using the LightCycler FastStart DNA Master SYRB Green I kit (Roche Molecular Biochemicals). Primer sequences for *18S* (used as reference gene), *HER-2,* and *PDGFRA* were designed by Primer Premier 5.0 (*18S*: forward, AGTTGGTGGAGCGATTTG; reverse, TTGCTCAATCTCGGTGG; *HER-2*: forward, CCTCATCAAGCGACGGC; reverse, CTGGTGGAGCCGCTGA; *PDGFRA*: forward, TGCTGTAGAAAGGCAAAGG; reverse, AACTGGCTGAAGGTGGGT). The primer sequences for analyzing the remaining 6 genes have been described previously.[9,13]

PCR was carried out as follows: after an initial 10-minute preincubation step at 95°C, 45 amplification cycles were run, each consisting of 95°C for 10 seconds, 58°C to 61°C for 10 seconds, and 72°C for 100 seconds. The relative amounts of genes were compared with the reference gene (*18S*) and calculated with LightCycler Relative Quantification Software (Roche Molecular Biochemicals). To confirm the specificity of the amplification signal, we considered the gene dissociation curve in each case.

To obtain gene dosages, the *18S* gene was used as a control and the corrected gene dosage for each gene was obtained based on the assumption that the gene ratio in normal tissue was 1.0 (studied gene/reference gene [*18S*] = 1). A ratio of more than 2.0 was regarded as positive for amplification.[18,19] Although ratio values of less than 0.5 might be compatible with gene deletion, this aspect was not considered in the present study.

Results

Of 40 samples, 23 (58%) were positive for amplification in real-time PCR in at least 1 gene. By histopathologic grade, 14 (67%) of 21 grade II oligodendrogliomas, 6 (46%) of 13 grade III anaplastic oligodendrogliomas, and 3 (50%) of 6 mixed oligoastrocytomas were positive for amplification.

For genes located at 12q13-14, *CDK4* was amplified in 12 (30%) of 40 samples, with similar amplification frequencies for grade II and III oligodendrogliomas (6/21 [29%] and 3/13 [23%], respectively) **Image 1**. Half of the mixed tumors showed amplification of this gene. *MDM2* amplification was detected in 12 (30%) of 40 cases, the same frequency as for *CDK4*. Of 21 grade II oligodendrogliomas, 8 (38%)

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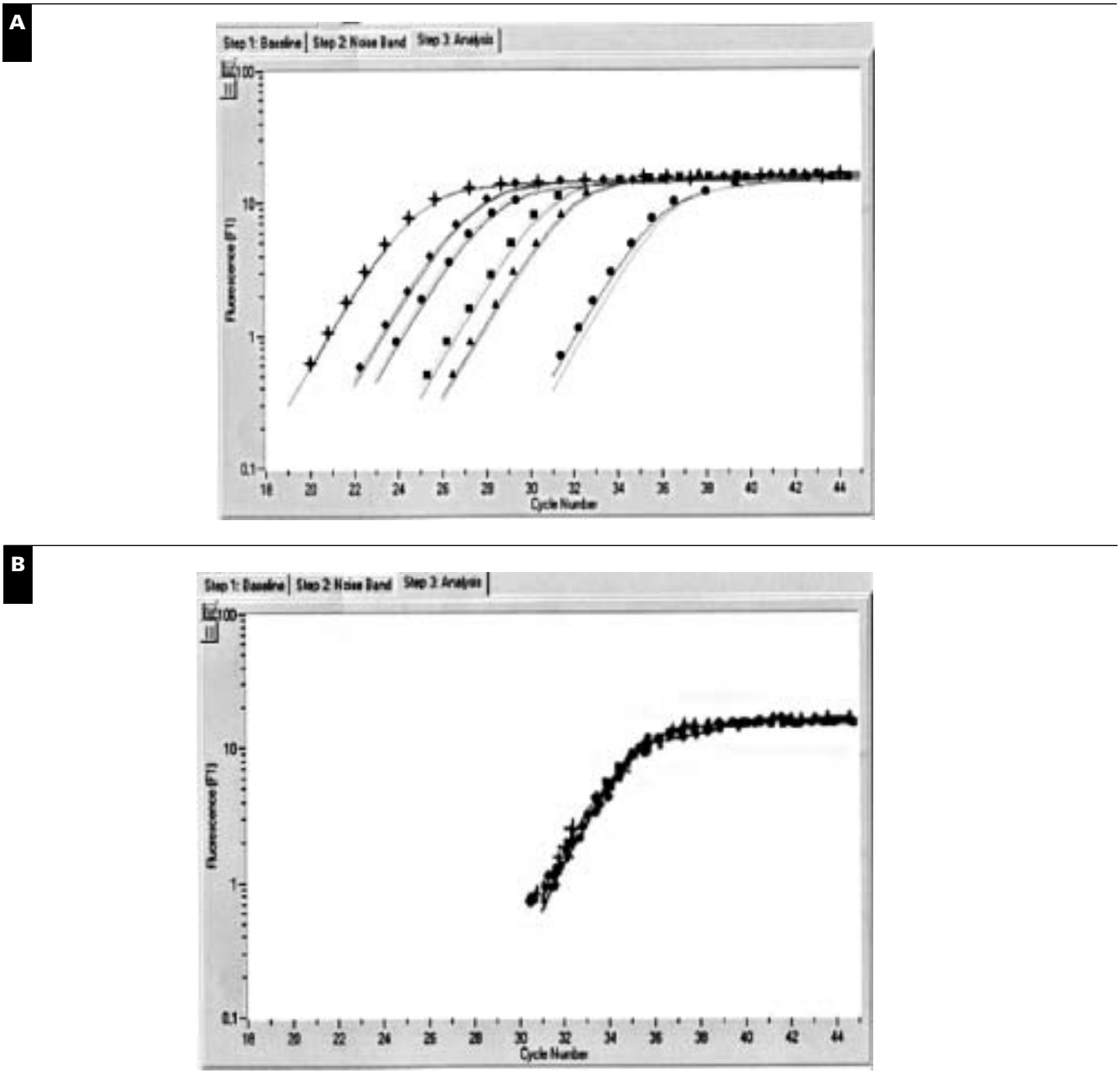

Image 1 Real-time quantitative polymerase chain reaction amplification of *CDK4* (target) and *18S* (reference). The curves show sample amplification (duplicated). The target gene increases in different samples (**A**), whereas the control gene is amplified equally in all samples (**B**). Sample O-10 (circles) is negative for *CDK4* DNA overamplification. Samples O-15 (triangles), AO-22 (squares), AO-139 (ovals), O-136 (diamonds), and O-13 (+) are positive for *CDK4* DNA overamplification. AO, anaplastic oligodendroglioma; O, low-grade oligodendroglioma.

were positive for *MDM2* amplification, and 3 (23%) of 13 grade III tumors were positive for this gene. *MDM2* and *CDK4* were coamplified in 7 samples, including 4 of grade II, 2 of grade III, and 1 mixed oligoastrocytoma.

Four genes located at 1q32 were analyzed. A similar amplification frequency was found in grade II and grade III tumors for *REN1* (5/21 [24%] and 3/13 [23%], respectively), but none of the mixed sample cases showed this aberration. In contrast, *GAC1* amplification was detected in at least 30% of cases in a low-grade or anaplastic form, whereas *ELF3* amplification was found in 3 (14%) of 21 grade II and 3 (23%) of 13 grade III oligodendrogliomas. *MDM4* was the least frequently affected gene; 1 low-grade and 1 anaplastic oligodendroglioma and 2 mixed tumors displayed this amplification.

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Indeed, *MDM4* was coamplified with *ELF3* or *REN1* in 1 sample each (AOA-6 and AO-139, respectively). No coamplification of all 4 genes at 1q32 (*ELF3, REN1, GAC1,* and *MDM4*) was found. However, 3 genes from this location (*ELF3, REN1,* and *GAC1*) showed coamplification in 2 samples (O-136, AO-133). Coamplification of 2 genes (*REN1* and *GAC1*) was identified in 6 samples, including 3 grade II oligodendrogliomas (14%) and 3 grade III anaplastic oligodendrogliomas (23%). Only 1 anaplastic oligodendroglioma (AO-139) showed *REN1* and *MDM4* coamplification, and 1 oligoastrocytoma (AOA-6) showed *ELF3* and *MDM4* coamplification.

HER-2 was positive for amplification in 6 (15%) of 40 samples, including 4 grade II (19%) and 2 grade III (15%) cases but none of the mixed tumors.

PDGFRA amplification was observed in 2 (10%) of 21 pure oligodendrogliomas, whereas 2 (33%) of 6 mixed anaplastic tumors displayed this anomaly.

Table 1 shows the gene dosages for all 8 genes studied. The amplification rates do not vary statistically between grade II and III oligodendrogliomas ($P > .05$), and the genetic amplification levels are not higher in the grade II tumors. Four genes in our study (*CDK4, MDM2, REN1,* and *GAC1*) were amplified in at least 20% of low-grade and anaplastic tumors. Accordingly, amplification of these genes might be associated nonrandomly with the development of oligodendroglioma.

Discussion

Amplification generally results in enhanced levels of the products encoded by the amplified gene. This alteration is one of the mechanisms by which cells synthesize specific gene products in excessive amounts.[8]

Proto-oncogene amplification is an important event in glioma development,[8] and several of these genes might be involved in the pathogenesis of these tumor types owing to their role in cell cycle regulation (*CDK4* and *MDM2*), chromosomal location (1q32: *MDM4, GAC1, REN1,* and *ELF3*), or function as growth factor receptors (*HER-2* and *PDGFRA*).

CDK4 belongs to the cyclin-dependent kinase family that, together with other cyclin proteins, has an important role in the regulation of the cell cycle.[20] Previous studies have shown *CDK4* amplification in 11% of glioblastomas,[21] and this alteration is rare in lower grade forms.[22] However, our data show similar *CDK4* amplification frequencies in grades II and III (29% [6/21] and 23% [3/13], respectively), and oligoastrocytomas showed a 50% frequency of this anomaly.

MDM2 amplification and overexpression have been detected in several tumor types, including malignant gliomas.[8,13,23] This alteration could be an alternative mechanism that might induce TP53 protein inactivation, inhibiting its oncosuppressor function and, therefore, producing cellular growth alterations.[24] Grade III oligodendrogliomas in our series showed *MDM2* amplification in 23% of samples (3/13). These data contrast with previously reported results in other nervous system tumor subtypes that found 8% to 10% amplification rates.[23] These discrepant findings may be due to the fact that most previous studies on the subject were performed on glioblastoma (WHO grade IV) using the Southern blot technique. In contrast, we analyzed grade II and III oligodendrogliomas with quantitative PCR, demonstrated to be a more sensitive technique.[8,13,23]

CDK4 and *MDM2* coamplification was found in 7 (18%) of 40 samples, in agreement with other studies.[13,25] The frequency of amplification in both genes separately was 30% (12/40), and their gene dosages were similar in almost all samples (eg, O-13 shows ratios for *CDK4* of 12.42 and for *MDM2* of 10.09). Thus, our results suggest that in certain samples, a *CDK4/MDM2* amplicon might be involved in the pathogenesis of oligodendroglioma and that the amplicon already would be present in the low-grade forms; we detected coamplification in 4 low-grade tumors.

MDM4, located at 1q32, encodes a TP53-binding protein with structural homology to *MDM2.* Binding of *MDM4* to TP53 inhibits TP53-dependent growth control in malignant gliomas.[12] Although the difference was not statistically significant because of our sample size, our data show a higher *MDM4* amplification frequency in grade III than in grade II oligodendrogliomas (8% [1/13] and 5% [1/21], respectively). This fact suggests that this gene could be involved in oligodendroglioma progression, in agreement with previously reported data.[26]

The *GAC1* gene, located on 1q32.1, can be amplified and its protein overexpressed in malignant gliomas.[27] It encodes a product belonging to the leucine-rich repeat superfamily and shares structural homology with other proteins involved in cellular adhesion molecules or protein receptors.[27] In our series, *GAC1* and *MDM4* coamplification occurred in 3 (8%) of 40 samples, in contrast with findings by Riemenschneider et al,[13] in which all samples with *MDM4* amplification also showed *GAC1* amplification. *GAC1* function in oligodendrogliomas is unclear at present, but according to our data, it could be involved as a nonrandom and early molecular abnormality in oligodendroglioma development; we detected *GAC1* amplification in 13 (33%) of the 40 tumors.

In our study, *REN1* amplification frequency was almost the same in grade II and III tumors (24% [5/21] and 23% [3/13], respectively), whereas Riemenschneider et al[13] found no *REN1* amplification. Two of our cases (O-15 and O-136) showed *REN1* and *GAC1* coamplification with almost equal ratio values (*REN1*: 2.59 and 6.76; *GAC1*: 2.13 and 6.93, respectively); therefore, these genes seem to arise from the same amplicon in some samples. The biologic significance of *REN1* amplification in oligodendrogliomas is not yet understood. The

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Table 1
Gene Ratios for Genes Amplified by Quantitative Polymerase Chain Reaction and Their LOH Status at 1p and 19q*

	12q13-14		1q32				17q21	4q11	LOH	
Sample No.	*CDK4*	*MDM2*	*REN1*	*GAC1*	*ELF3*	*MDM4*	*HER-2*	*PDGFRA*	1p	19q
O-2	1.92	3.57	0.94	5.08	2.10	1.50	3.00	1.19	+	+
O-5	0.88	1.46	0.95	1.78	0.63	0.85	2.14	0.72	+	+
O-8	0.81	2.18	1.49	0.83	1.12	0.92	1.30	1.96	+	+
O-9t1	0.54	1.46	0.56	0.97	1.21	1.32	1.70	1.99	+	+
O-10	0.36	1.45	0.60	1.38	0.51	0.32	0.53	1.14	+	–
O-12	1.47	0.40	0.29	1.18	1.20	1.08	0.63	1.34	+	–
O-13	12.42	10.09	0.84	3.41	1.24	1.32	0.43	0.73	–	–
O-15	2.20	3.45	2.59	2.13	1.93	1.24	1.84	1.35	+	–
O-16	1.17	0.80	0.52	0.47	0.47	0.43	0.72	1.69	+	+
O-21	0.51	2.56	0.61	2.51	0.55	1.25	1.42	1.56	+	+
O-24	1.02	2.86	2.29	1.66	1.98	1.91	0.81	2.49	+	+
O-25	0.84	0.92	0.34	0.42	0.60	1.24	1.69	0.51	–	+
O-26	1.23	1.48	0.91	4.50	0.44	2.55	2.69	1.46	+	+
O-131	0.93	1.94	3.99	2.80	1.30	1.28	0.52	1.01	+	+
O-132	2.14	0.43	0.94	1.27	0.63	0.89	0.92	1.42	–	–
O-134	2.38	1.63	1.39	0.83	0.31	1.41	1.08	0.65	+	+
O-136	8.00	3.77	6.76	6.93	2.30	1.69	1.17	1.18	+	+
O-137	0.98	1.42	1.40	4.94	3.55	1.49	4.44	3.56	–	–
O-138	2.50	2.55	3.38	0.87	1.18	0.51	0.78	0.78	–	+
O-140	1.22	1.31	1.15	0.79	1.89	1.36	1.12	0.83	–	–
O-144	1.27	1.52	0.32	0.77	1.12	1.12	1.86	0.53	–	–
No. (%) amplified (n = 21)	6 (29)	8 (38)	5 (24)	8 (38)	3 (14)	1 (5)	4 (19)	2 (10)	—	—
AO-3	1.65	0.88	0.91	6.92	7.90	1.65	0.56	0.57	+	+
AO-9t2	0.60	1.50	0.57	0.60	1.19	1.26	1.68	1.92	+	+
AO-11	0.57	0.87	0.58	1.34	0.56	1.31	0.55	1.35	+	+
AO-17	1.34	0.85	0.86	1.39	1.29	1.27	0.57	1.02	+	+
AO-22	3.10	0.45	2.77	2.34	0.72	1.87	1.78	0.59	–	+
AO-23	0.79	0.30	0.61	1.02	1.42	1.03	1.19	1.00	+	–
AO-27	1.02	1.37	1.04	0.61	0.44	0.51	0.97	1.52	–	+
AO-133	2.27	6.92	2.79	3.27	4.10	1.63	3.72	1.18	+	+
AO-139	5.19	4.48	5.50	3.41	1.30	2.75	2.59	0.65	+	+
AO-141	1.58	2.17	0.80	0.89	2.20	1.81	1.12	0.85	+	+
AO-142	1.56	0.98	0.53	0.58	1.26	0.87	1.25	1.56	+	+
AO-143	1.40	0.76	0.71	1.31	1.23	0.94	0.92	2.99	+	+
AO-145	0.51	0.58	1.20	1.36	0.45	0.57	1.34	0.36	–	–
No. (%) amplified (n = 13)	3 (23)	3 (23)	3 (23)	4 (31)	3 (23)	1 (8)	2 (15)	1 (8)	—	—
OA-1	2.28	1.58	0.48	1.36	1.10	1.45	1.52	0.70	+	+
OA-4	0.76	1.49	0.99	0.62	0.56	0.46	0.86	0.55	+	+
OA-7	1.15	0.53	0.46	0.59	0.63	0.42	1.20	0.44	–	+
OA-28	1.14	1.18	0.51	1.02	1.30	1.24	0.62	0.57	–	–
AOA-6	2.22	0.52	0.72	1.26	3.04	2.65	0.93	4.51	+	+
AOA-135	2.49	3.96	0.67	2.85	1.31	2.16	0.75	4.09	+	–
No. (%) amplified (n = 6)	3 (50)	1 (17)	0 (0)	1 (17)	1 (17)	2 (33)	0 (0)	2 (33)	—	—
Total amplified (%) (n = 40)	12 (30)	12 (30)	8 (20)	13 (33)	7 (18)	4 (10)	6 (15)	5 (13)	—	—

AO, anaplastic oligodendroglioma (WHO grade III); *CDK4,* cyclin-dependent kinase-4 gene; LOH, loss of heterozygosity; O, low-grade oligodendroglioma (WHO grade II); OA and AOA, mixed oligoastrocytoma (WHO grades II and III, respectively); *PDGFRA,* platelet-derived growth factor receptor-A gene; WHO, World Health Organization; +, positive for LOH; –, negative for LOH.

* Ratios were determined as studied gene/reference gene; ratios >2.0 were considered positive for amplification. The range of the experimental error mean was ± 0.55 based on examination of normal control and repeated samples. LOH data are according to a report by Alonso et al.[7]

renin-angiotensin system is one of the main homeostasis-regulating mechanisms, and its stimulation of angiogenesis could be involved in tumoral cell blood supply.[28] The possibility exists that increased expression of renin could provide a growth advantage by promoting neovascularization in oligodendrogliomas.

The fourth gene we studied at 1q32.2 was *ELF3,* 1 of 30 Ets transcription factor family members. ELF3 is expressed in many but not all epithelial cells and has a regulating genic role during epidermal terminal differentiation.[29] *ELF3* could have a critical role as transcriptional controller of stringently regulated genes, such as those involved in tissue development, differentiation, angiogenesis, cellular cycle control, and cell proliferation, and functions such as transcriptional repression and enhancement.[30,31] Although its relation with nervous system tumors is unknown, previous studies on mouse

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mammary tissues demonstrated that altered *ELF3* expression can be involved in tumor progression.[32] Our data show a higher *ELF3* amplification frequency in grade III than in grade II tumors, which might indicate some effect on oligodendroglial progression; expression studies will be useful to demonstrate whether amplification in this gene has a direct consequence in this kind of glial tumor.

We found *ELF3* and *GAC1* coamplification in 3 samples (O-137, AO-3, and AO-133) that showed very similar amplification ratios (*GAC1*: 4.94, 6.92, 3.27; and *ELF3*: 3.55, 7.9, 4.1, respectively). Coamplification would be consistent with the evidence of an amplicon involving these 1q32 genes in these samples.

The *HER-2* gene, mapped at 17q21, is one of the most studied genes in cancer.[15] *HER-2* encodes a transmembrane glycoprotein that belongs to the *EGFR* family.[14] This protein has intrinsic tyrosine kinase activities and can be involved in the regulation of a variety of vital functions, such as cell growth, differentiation, and apoptosis.[33] Previous data showed that amplification is associated with breast tumors that metastasize to the nervous system.[34] In fact, HER-2 overexpression has been proposed as a molecular marker for predicting such metastases.[34] No previous data on *HER-2* amplification in gliomas are available, and our findings show that this gene is amplified in low-grade and anaplastic tumors at significant rates (19% [4/21] and 15% [2/13], respectively).

Gliomas frequently have mutation or expression abnormalities that can activate tyrosine kinase signal pathway genes such as *PDGFRA*.[35] This gene seems to have an important role in normal development and tumorigenesis in the nervous system.[35] In several high-grade oligodendrogliomas, *PDGFRA* amplification suggests the possibility of an autocrine inhibition of PDGF and its receptors.[36] We found *PDGFRA* amplification in low-grade (2/21 [10%]) and anaplastic tumors (1/13 [8%]), in contrast with an earlier study reporting that amplification occurred almost exclusively in grade III oligodendrogliomas.[17] In our mixed tumors, *PDGFRA* amplification was an important alteration because it was present in 33% of the cases (2/6, both diagnosed as anaplastic grade III), although a larger number of samples is necessary to confirm *PDGFRA* action in this kind of tumor. In contrast with the suggestion of Smith et al,[17] 3 of 5 samples in our series showed *PDGFRA* amplification and losses at 1p and 19q, suggesting that these alterations are not necessarily exclusive.

The present study shows that oligodendrogliomas are characterized by amplification of multiple genes from chromosomal segments 12q13-q14 and 1q32 and the *HER-2* and *PDGFRA* genes. It is interesting that gene amplification already is present in low-grade samples, suggesting that this molecular abnormality might be a relatively early step in the development of oligodendrogliomas.

From the Departments of [1]Experimental Surgery, Molecular Oncogenetics Laboratory, Research Unit, and [3]Neurosurgery and [5]Pathology, Hospital Universitario La Paz, Madrid, Spain; [2]Neurosurgery, Hospital del Río Hortega, Valladolid, Spain; [4]Neurosurgery, Clínica Puerta de Hierro, Madrid, Spain; and [6]Pathology, Fundación Jiménez Diaz, Madrid, Spain.

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Address reprint requests to Dr Rey: Dept of C. Experimental, Laboratorio de Oncogenética Molecular, Unidad de Investigación, Hospital Universitario La Paz. Paseo de la Castellana 261, 28046 Madrid, Spain.

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