The Early Growth Response Gene *EGR-1* Behaves as a Suppressor Gene That Is Down-Regulated Independent of ARF/Mdm2 but not p53 Alterations in Fresh Human Gliomas[1]

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ABSTRACT

Purpose: ***EGR-1* is an immediate early gene with diverse functions that include the suppression of growth. *EGR-1* is down-regulated many cancer cell types, suggesting a tumor suppressor role, and may critically involve the p53 pathway. The aim of this work was to measure the expression of *EGR-1* and the p16/INK4a/ARF-Mdm2-p53 pathway status in fresh human gliomas.**

***Experimental Design*: Thirty-one human gliomas with different grades of malignancy were investigated for Egr-1 mRNA and the protein expression, frequency, and spectrum of *p53* gene mutations, *mdm2* gene amplification, and *p16/INK4a/ARF* allele loss.**

***Results*: The amplification of *Mdm2* and the deletion of the *p16/INK4a* gene was found in 3 and 5 cases, respectively, whereas mutations of *p53*, including two novel mutations, were observed in 10 other cases. The three types of changes occurred strictly mutually exclusively, emphasizing that these genes operate in a common pathway critical to glioma progression. EGR-1 mRNA was significantly down-regulated in astrocytomas (14.7 ± 5.1%) and in glioblastomas (33.6 ± 10.0%) *versus* normal brain. Overall, EGR-1 mRNA was strongly suppressed (average, 15.2 ± 13.9%) in 27 of 31 cases (87%), independent of changes in *p16/INK4a/ARF* and *Mdm2*; whereas 4 of 31 cases with residual EGR-1 expression as well as the highest EGR-1 variance segregated with *p53* mutations. Immunohistochemical analyses confirmed the suppression of EGR-1 protein.**

***Conclusions*: These results indicate that EGR-1 is commonly suppressed in gliomas independent of *p16/INK4a/ARF* and *Mdm2* and that suppression is less crucial in tumors bearing *p53* mutations, and these results implicate an EGR-1 growth regulatory mechanism as a target of inactivation during tumor progression.**

INTRODUCTION

Several important genes with tumor suppressive functions have been implicated for the pathogenesis of astrocytic neoplasms (1, 2). Mutational inactivation of *p53* is found in up to 50% of tumors, and the application of gene transfer techniques for replacement of function is presently under experimental evaluation (3). For an appreciable fraction of gliomas, however, the finding of responsible genes is still elusive.

EGR-1 (also known as *NGFI-A*, *TIS8*, *Krox-24*, and *Zif268*) is a member of the immediate early genes family and encodes a nuclear phosphoprotein involved in the regulation of cell growth and differentiation in response to signals such as mitogens, growth factors, and stress stimuli (4–7). However, in other circumstances, *EGR-1* is induced very early in the apoptotic process (8), where it mediates the activation of downstream regulatory genes such as *p53* and *tumor necrosis factor* α (9, 10).

In contrast with these functions, previous studies have shown that *EGR-1* is down-regulated in several types of neoplasia, suggesting a role as a tumor suppressor gene in analogy with WT-1, another family member which binds to a similar DNA motif (11, 12). For example, EGR-1 has been found to be decreased or undetectable in human breast and small cell lung tumors (13, 14) as well as in an array of tumor cell lines (11, 15, 16). Gene deletion or mutations have also been reported in sporadic cancer cases (17). Moreover, reexpression of EGR-1 suppresses the growth of transformed cells both in soft agar and in athymic mice (16). This is at least partially related to the

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induction of TGF-β1[4] expression—a factor with an important role in the progression of gliomas (18, 19). Similarly, studies with antisense vectors indicated that the transformed phenotype is enhanced by the inhibition of EGR-1 expression (20). These studies suggest a consistent growth suppression role for EGR-1 in cells of neuroectodermal origin. Here, we directly test this hypothesis by examination of the expression of EGR-1 in fresh surgical specimens of glioblastomas and normal tissue.

EGR-1 has been implicated in the regulation of p53, thereby providing an additional explanation for a suppressor role (9, 10, 21, 22). The function of p53, in turn, is determined in part by the Mdm2 protein and by an alternate reading frame (ARF or p14ARF) product of the *p16/INK4a* locus. ARF binds to Mdm2, allowing active p53 to accumulate in the nucleoplasm (23). Indeed, homozygous deletion of the murine *ARF* gene in mice leads to a similar phenotype as for inactivation of p53, further indicating that ARF and p53 function in the same biochemical pathway (reviewed in Ref. 24). Thus, deletion of *ARF* or overexpression of *Mdm2* may be functionally redundant means of disrupting the ARF-Mdm2-p53 pathway. In ~75% of B-cell lymphomas that develop in *myc*-transgenic mice, there occurs either a mutation in *p53*, deletion of *ARF*, or overexpression of *Mdm2*, but not more than one of these alterations (25). Similarly, in humans, *p53* is itself mutated in >50% of cancers, whereas deletion of *ARF* and amplification or overexpression of *Mdm2* occur in a high fraction of the remaining cases (24). This mutational pattern is commonly manifested in human glioblastomas as well (26–28). Therefore, to examine the relationship between EGR-1 and p53, we also examined the genetic status of p16INK4a, Mdm2, and p53 in the primary glioblastomas.

We find that EGR-1 expression was strongly reduced in fresh, surgically derived brain tumors compared with normal brain tissue, where the basal expression is high (reviewed in Ref. 29). EGR-1 was suppressed in >87% of the cases independent of whether alterations in *p16/INK4a* and *Mdm2* were present but significantly less suppressed and more variable in tumors bearing *p53* mutations. These results indicate that loss of *EGR-1* expression may provide an important marker of glial cell malignancy.

MATERIALS AND METHODS

Tissue Samples. We examined 31 tumors from astrocytoma (astrocytic tumors grades I-III) or glioblastoma (astrocytic tumors grade IV) patients at Instituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Neuromed, Pozzilli, Italy, an affiliated clinic of the University of Rome "La Sapienza." Donor patients were restricted to those not treated previously with chemotherapy or radiation therapy. Tumor samples were collected from zones free of any necrotic tissue at the time of the initial "debulking" surgery and processed immediately. One aliquot of each tissue was fixed in 10% formalin for histological analyses and grading. Another aliquot was frozen at −70°C for DNA, RNA, and protein extraction. A third aliquot was fixed overnight in 4% paraformaldehyde (pH 7.2) and used for paraffin embedding. The remainder was frozen in liquid nitrogen. Several human specimens of normal white and gray matter were obtained either from adult patients that were being treated surgically for refractive drug-resistant epilepsy or within 8 h postmortem from subjects of accidental death. A sample of human total RNA from normal adult brain was purchased from Invitrogen (Carlsbad, CA). Primary astrocytes were prepared from the whole brain of neonatal Wistar rats as described (30).

Regulatory Affairs. The Institutional Ethics Board approved the experimental design and scope of this study. All subjects provided a written informed consent for this study. Only tissue excess to diagnostic and patient management needs was made available for research.

Isolation and Northern Analysis of Total RNA. Total RNA was extracted from tumor pieces (~50 mg) using the Ultraspec RNA isolation system (Biotecx Laboratories, Houston, TX). For Northern analysis, 20 μg of denaturated RNA was used for each sample and processed as described previously (15). Levels of *EGR-1* expression in control and cancer patients were expressed as means ± SE and compared using the *t* test, ANOVA, and nonparametric Mann-Whitney *U* test. All tests were two-sided, with a critical level for significance defined as $P < 0.05$.

DNA Extraction and Analysis. For Southern analysis, 20 μg of high-molecular weight DNA was digested with 50 units of TaqI, electrophoretically separated in 0.8% agarose gel, and transferred to nylon membranes, as above. Membranes were hybridized with DNA probes labeled with [^{32}P]dATP by random priming. The GAPDH probe was a 1.3-kb, full-length cDNA clone encoding rat GAPDH (31). A 1.6kb *Bgl*II fragment from pCMV-*EGR-1* plasmid was the probe for EGR-1 (32), corresponding to the coding region of the mouse cDNA. The DNA probes for *MTS1/p16*, a 514-bp fragment corresponding to exon 2 of the *MTS1* gene, and for *Mdm2*, bases 53–653 of the human *Mdm2* cDNA sequence, were generated by PCR amplification as described (33, 34). The identity of the generated probes was corroborated by restriction-enzyme analysis. The probe for *HTSHR* was the 2.5kb *Eco*RI fragment from the pBabeTSHR plasmid (35) corresponding to the full-length cDNA for HTSHR. The *Mdm2*, *MTS1*, and *HTSHR* probes identify 8.0- and 5.5-kb, 3.3-kb, and 4.2- and 1.9-kb bands, respectively.

DNA Sequencing of *p53* Exons 5–9. For analysis of *p53* mutations, three DNA fragments (containing exons 5 and 6, exon 7, and exons 8 and 9, respectively) were PCR-amplified from genomic DNA by using primers described previously (36).

Direct sequencing was performed with a ABI PRISM 377 DNA Sequencer using the DNA Sequencing kit, Big Dye Terminator (PE Applied Biosystems, Inc., Boston, MA) according to the manufacturer's instructions. The results were analyzed by means of the ABI sequencing analysis software.

Densitometric Analysis and Allele Dosage. Densitometric scans of Northern and Southern results were elaborated using the NIH Image software.[5] For Northern analysis, the

[4] The abbreviations used are: TGF-β1, transforming growth factor β1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HTSHR, human thyrotropin receptor; NBA, astrocytes from newborn rat brain; WM, human white matter; TB, human total brain; GFAP, Glial fibrillary acidic protein; MTS = 1, multiple tumor suppressor = 1.

[5] Available on the Internet at: http://rsb.info.nih.gov/nih-image.

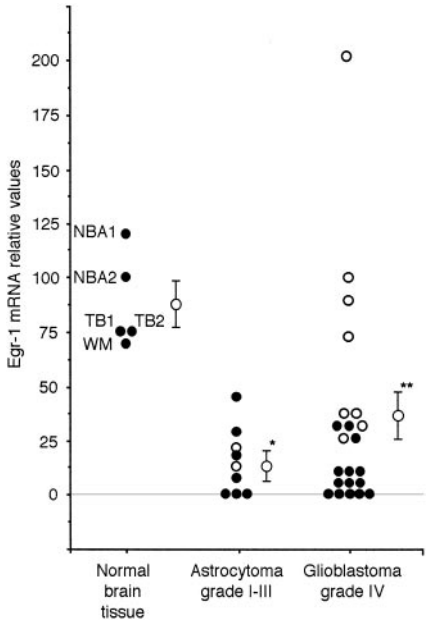

Fig. 1 Quantification of EGR-1 expression in fresh glioma biopsies by Northern blot as compared with normal brain tissue. EGR-1 mRNA levels are down-regulated in glioma. The EGR-1 mRNA levels were determined by densitometry and expressed as a percentage of normal (*NBA2*). Three different groups of data are shown: normal brain (5 cases), astrocytoma (9 cases), glioblastoma (22 cases). ○, cases carrying mutated copies of the *p53* gene. For each group of data, the mean ± SE is represented. The mean values of astrocytomas [$P < 0.005$ (*)] and glioblastomas [$P < 0.01$ (**)] differ significantly from normal tissue.

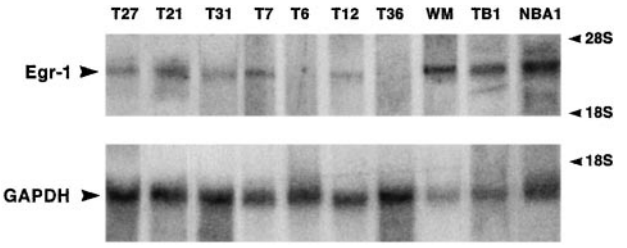

Fig. 2 Northern analysis of EGR-1 expression in representative cases of fresh human malignant glioma compared with normal controls. As a nucleic acid probe for EGR-1 RNA, a 1.6-kb BglII fragment from the cDNA coding sequence of the mouse gene was used. This fragment shows an 87% sequence similarity to the human homologue. Representative tumor biopsies are indicated as *T* (with numeral). Three different controls are included in this blot: one sample of NBA (*NBA1*) and WM (*WM*) and one of TB (*TB1*). These same RNAs were included as cross-reference controls in each independent experiment. Blots were rehybridized with a GAPDH probe. Exposure times for the EGR-1- and GAPDH-hybridized filter shown are 72 and 12 h, respectively.

EGR-1 signals were normalized to the expression of the *GAPDH* gene of each sample, probed on the same membranes. Allele dosage was measured and calculated as described (34). Hybridization signals were normalized to the values observed at the control locus *HTSHR* on the same membrane. *Mdm2* amplification is defined as at least five copies of gene/cell. Loss of heterozygosity at the *p16INK4a* locus was defined as a >40% decrease in the densitometry value (37).

Immunohistochemistry. A rabbit polyclonal antibody against EGR-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse monoclonal antibody against GFAP (Sigma Chemical Co., St. Louis, MO) and p53 (PharMingen, Heidelberg, Germany) were used for staining. Peroxidase-conjugated secondary antibodies were localized with the aid of an ABC Universal Quick kit (Vector Laboratories, Burlingame, CA) following the manufacturer suggested procedure.

RESULTS

EGR-1 Is Down-Regulated in Malignant Brain Tumors. Thirty-one fresh glioma biopsies were obtained from patients diagnosed with astrocytoma ($n = 9$) or with glioblastoma ($n = 22$) and frozen in liquid nitrogen immediately upon resection. Tumor staging and grade were established according to WHO classification. To evaluate *EGR-1* expression levels, three different normal sources of mRNA, including fresh preparations of NBA and WM, were analyzed. In addition, a commercial RNA from TB was investigated. Each tumor sample was evaluated at least twice and normalized to the level of *GAPDH* expression of the same sample. All RNAs of normal brain investigated exhibited a steady-state level of *EGR-1* expression within a close range of values (Fig. 1). As a reference value for basal *EGR-1* steady-state mRNA level, the intermediate level of expression of NBA2 was taken as 100%.

Steady-state *EGR-1* mRNA levels are significantly down-regulated in both astrocytomas and glioblastoma groups compared with normal tissue (Fig. 1). Mean values (± SE) are 88.0 (± 9.6) in normal brain tissue, 14.7 (± 5.1) in astrocytomas ($P < 0.005$ *versus* normal), and 33.6 (± 10.0) for the 22 glioblastoma cases ($P < 0.01$ *versus* normal). Most glioblastomas, 87%, are tightly clustered within an expression level of less than one-fourth of the reference tissue. However, four glioblastoma cases exhibited mRNA levels comparable with or even greater than those of normal tissue, suggesting a bimodal distribution within the glioblastoma multiforme group. Other distinguishing features of this high expression group are noted below. Examples of Northern analysis for the various tumors and normal brain samples are shown in Fig. 2.

We also carried out Western analysis (data not shown), and we observed that basal EGR-1 protein is undetectable in most cases, similar to the observations of mRNA. However, several exceptions that exhibit high levels of EGR-1 protein, such as tumor cases T15 and T36 were noted. Thus, the circumstances associated with EGR-1 expression were investigated in greater detail.

EGR-1 protein expression and localization in normal human brain tissue and in the gliomas was investigated by immunocytochemistry (Fig. 3). Similar to mRNA, EGR-1 was less apparent in tumors compared with normal astrocyte populations. In normal subpial and white matter astrocytes, EGR-1 expression was readily apparent and widely distributed. Within cells, EGR-1 was localized to the cell nucleus. Within nuclei, EGR-1 was either diffuse or limited to nuclear membrane in most cases. Cortical neurons were strongly positive with a nuclear localization similar to that of astro-

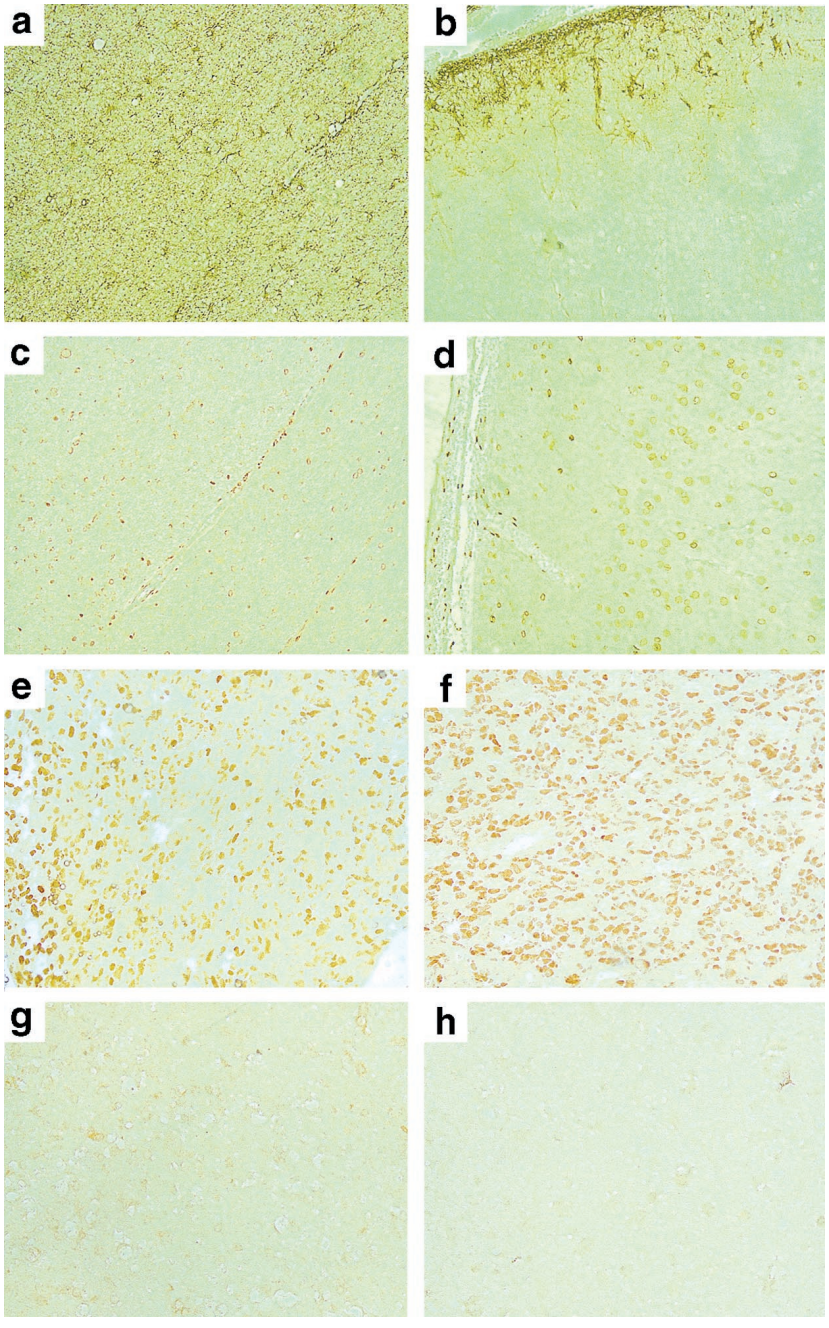

Fig. 3 Localization of EGR-1 in normal human brain tissue and in fresh human malignant gliomas with wild-type or mutant *p53*. GFAP (*a* and *b*) and EGR-1 (*c* and *d*) are stained by immunohistochemistry in human brain sections from white (*a* and *c*) and gray matter (*b* and *d*). EGR-1 protein is associated preferentially with nuclear membrane in both astrocytes from white and gray matter and neurons (*gray matter*). Expression pattern of EGR-1 and p53 in tumor astrocytes. EGR-1 (*e* and *g*) and p53 (*f* and *h*) are stained by immunohistochemistry in contiguous sections from *p53*-mutated (*e* and *f*) and wild-type tumors (*g* and *h*).

cytes (Fig. 3, *c* and *d*). GFAP staining was performed to identify the astrocyte population in gray and white matter (Fig. 3, *a* and *b*). To our knowledge, this is the first observation of EGR-1 protein in normal human brain tissue.

The analysis of the pattern of EGR-1 protein expression in tumors with varying levels of *EGR-1* mRNA showed that all tumor biopsies with levels of *EGR-1* RNA expression from 15–200% relative to NBA2 (Fig. 1) stained positively for EGR-1 protein. However, the fraction of positive cells for each tumor ranged from around 13% to no more than 30%. Conversely, efforts to detect EGR-1 protein were, as a rule, negative in all sections from those tumors showing deficient mRNA expression, although the presence of a few EGR-1-positive cells, accounting for $<2\%$ of the total cell population, could be observed. Inasmuch as EGR-1 is highly expressed in normal brain and because gliomas are highly infiltrating tumors, it might not be infrequent that normal cells are found mixed within the tumor population. For these reasons, we suspect that the amount of RNA and protein found in the tumors investigated here might be overestimated. Moreover, we cannot rule out that stable EGR-1 protein derived from the normal cells that occur in low-grade

Table 1 Summary of mutations found in a series of 31 astrocytic tumors

Case	Diagnosis	Exon	Codon	*p53* mutation	Substitution		*Mdm2* DNA	Egr-1 RNA (% normal)	*P16* genotype
T26	G[a]	6	213	C→G	Arg→Gly	N	200	+/+	
T21	G	5	152	C→T	Pro→leu		N	100	+/+
T28	G	7	241	C→T	Ser→Phe	I[b]	N	90	+/+
T46	G	7	241	C→T	Ser→Phe	I	N	75	+/+
T2	A	6	216	G→A	Val→Met		N	12	+/+
T7	G	7	237	G→A	Met→Ile	I	N	30	+/+
T15	A	5	175	G→A	Arg→His	I	N	20	+/+
T33	G	8	278	C→T	Pro→Leu	I	N	36	+/+
T40	G	6	193	A→T	His→Leu	I	N	24	+/+
T48	G	8	266	G→C	Gly→Arg		N	35	+/+
								Av: <66>	
T27	G	No change					Amplified	30	+/+
T31	G	No change					Amplified	15	+/+
T45	G	No change					Amplified	13	+/+
								Av: <19>	
T1	G	No change					N	0	del/del
T3	G	No change					N	5	del/del
T11	G	No change					N	2	del/del
T16	G	No change					N	10	+/del
T36	G	No change					N	0	del/del
								Av: <3>	
T13	G	No change					N	25	+/+
T14	G	No change					N	0	+/+
T18	A	No change					N	30	+/+
T19	A	No change					N	10	+/+
T25	G	No change					N	8	+/+
T32	G	No change					N	12	+/+
T42	A	No change					N	0	+/+
T44	A	No change					N	45	+/+
T5	A	No change					N	0	+/+
T6	A	No change					N	0	+/+
T9	G	No change					N	30	+/+
T10	G	No change					N	0	+/+
T12	A	No change					N	15	+/+
							Av: <13>		

[a] G, glioma; A, astrocytoma; N, normal genotype; del, deleted allele; Av, mean percentage.
[b] I, mutation with probable inactivating function. These mutations fall into highly conserved structural motifs.

astrocytomas, such as cases T15 and T36, may cause the relatively high EGR-1 protein measurements observed in these cases. In conclusion, our *in situ* studies provide an explanation for the variation of EGR-1 protein expression in tumors that exhibit an overall suppression of mRNA and provide support for the general correlation of low levels of *EGR-1* mRNA in fresh tumor tissue and a parallel decrease in protein accumulation.

Frequency and Spectrum of *p53* Gene Mutations. Recent reports suggest that EGR-1 can induce the transcription of the *p53* gene leading to increased apoptosis of tumor cells and that, conversely, EGR-1 expression may be influenced by the level of p53 (9). These observations suggest, as one hypothesis, that suppression of EGR-1 may serve to augment the survival properties of astrocytic tumors by blocking, at least in part, the induction of p53 or by blocking a p53-related function. Therefore, we sought to determine whether *p53* gene status correlates with the EGR-1 expression level within the same samples.

The *p53* gene was characterized by examining the nucleotide sequences of exons 5–9 for the presence of mutations. Among the 31 patients examined, 10 mutations were found (2 astrocytomas and 8 glioblastomas), as summarized in Table 1. Briefly, two mutations were located in exon 5, three in exon 6, three in exon 7, and two in exon 8, all mutations resulting in an amino acid substitution. Three mutations were found in the L domain, and two in the LH domain. The L (codons 236–251) and LH (codons 163–195) domains are two loop-based elements that bind to DNA. Another mutation (T33) is located at LSH, a third loop-based *p53* domain. As reported in the literature, for most tumors, more than two-thirds of missense mutations are found in L, LH, or LSH (38).

More than half of the mutations found in our tumors probably inactivate the protein, because they fall into highly conserved structural motifs. These are indicated in Table 1 as "I." All mutations observed here were already found in tumors of different histological type,[6] except for T26 and T40, which bear mutations reported here for the first time.

[6] IARC TP53 Mutation Database, Internet address: www.IARC.FRX.

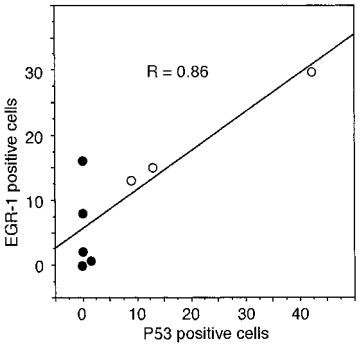

Fig. 4 Allele dosage for *Mdm2* and *p16INK4a* genes as determined by Southern analysis in representative tumor samples. *A*, 3 tumors (T45, T31, and T27) show amplified copies of the *Mdm2* gene. *B*, 5 tumors (T36, T24, T11, T3, and T1) show absence of both alleles of p16/MTS1. Tumor T16 shows single-allele deletion. *C*, the same filter as in *A* and *B* hybridized with a probe from the HTSHR gene (*HTSHR*), for signal normalization.

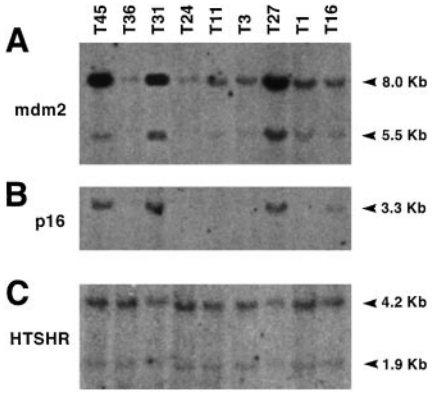

Fig. 5 *EGR-1* expression correlates with *p53* in mutated gliomas. Immunohistochemical evaluation of the percentage of *EGR-1*-positive cells in tissues from tumors carrying wild-type *p53* (●) or mutated copies of the gene (○). Staining of the p53 protein was demonstrated in all sections from the mutated tumors but not in sections from wild-type-*p53* tumors. The percentage of *EGR-1*-positive cells significantly overlapped the percentage of cells showing p53 protein accumulation in all of the three p53-mutated tumors investigated (correlation coefficient $r_{\text{Pearson}} = 0.86$; $P = 0.0067$).

Loss of *EGR-1* Expression Is Frequent in Tumors Carrying a Conserved p53 Pathway. By comparing the levels of *EGR-1* mRNA in the 21 tumors bearing wild-type copies of the *p53* gene with those present in samples with the mutated gene (Table 1), we noticed that *EGR-1* expression is significantly higher in *p53*-mutated tumors. The average values of *EGR-1* mRNA in the two groups were 11.9% (± 2.9) and 62.2% (± 18.1) compared with normal values, respectively ($P = 0.001$, according to Student's *t* test; and $P = 0.0002$, as evaluated by Mann-Whitney nonparametric *U* test). Moreover, in the subset of four tumors with mutated *p53* responsible for the bimodal expression of EGR-1 mRNA of the glioblastoma multiforme (Fig. 1), the average RNA expression is 116%, or very similar to that of normal tissue. Thus, the pattern of results is consistent with marked suppression of *EGR-1* mRNA levels in tumors bearing wild-type *p53*, whereas *EGR-1* is significantly less suppressed in the group bearing a mutated *p53* gene or not suppressed at all as in a subset of the tumors with a mutated *p53* gene.

These results were also confirmed by the evaluation of p53 intracellular protein levels, which are known to accumulate in cells carrying the mutated gene. Indeed, we examined both the expression of EGR-1 (Fig. 3*e*) and the accumulation of p53 protein by immunohistochemistry on contiguous tumor sections from tumor biopsies carrying mutations in the *p53* gene (Fig. 3*f*). The expression of EGR-1 and p53 was also examined in tumors carrying wild-type copies of the tumor suppressor gene as a control (Fig. 3, *g* and *h*). Labeled p53 protein was demonstrated in all sections from the tumors with mutated *p53* but not in sections from wild-type *p53*. The frequency of cells found in glioma specimens that were expressing p53 and/or EGR-1 is graphically reported in Fig. 4. EGR-1 expression significantly overlapped with p53 accumulation in all three examined tumors with mutated *p53* (correlation coefficient $r_{\text{Pearson}} = 0.86$; $P = 0.0067$). All of the four tumors that showed <10% EGR-1 positive cells had undetectable levels for EGR-1 mRNA expression. Thus both the mRNA analysis and immunohistochemical analysis indicated a consistent pattern of significantly elevated EGR-1 mRNA and protein expression in tumors lacking wild-type *p53*.

Determination of Alterations of Mdm2. The Mdm2 protein is an important p53 antagonist and, therefore, a potential indirect regulator of EGR-1 expression (24). Allele dosage determination of the *Mdm2* gene was undertaken for all 31 glioma biopsies by quantifying the Mdm2 Southern analyses hybridization intensities. Three tumor biopsies of glioblastoma (T27, T31, and T45) were found containing amplified copies of *Mdm2* (Fig. 5*A*). The allele dosage ratios ranged from ~25-fold to 50-fold in the case of T27. All three of these tumors expressed a wild-type *p53* gene (Table 1).

The finding of cases of amplified *Mdm2* genes only among tumors carrying wild-type copies of the *p53* gene suggests that the fraction of tumors showing impaired p53 function might be larger than could be estimated from the net number of cases showing *p53* mutations. Therefore, we also evaluated the differences for the EGR-1 mRNA values shown above by including within the group of patients with *p53* mutations the tumors with *Mdm2* mutations. Again, we confirmed that EGR-1 expression is significantly lower in nonmutated tumors: 10.7 ± 3.2% *versus* 52.3 ± 14.8% ($P = 0.003$, according to Student's *t* test; and $P = 0.0002$ as evaluated by Mann-Whitney nonparametric *U* test). In conclusion, all astrocytic tumors investigated here either were carrying mutations for the *p53* or *Mdm2* genes or presented strongly reduced levels of *EGR-1* gene expression (the EGR-1 mRNA mean value calculated from all but mutated cases is 13.5 ± 4.1%).

Analysis of Alterations of *p16* Gene. Deletions of *INK4a*, also known as *p16*, are commonly associated with deletion of the alternate reading frame product, ARF, an Mdm2 antagonist (39, 40). We investigated the *p16* gene in our panel of glioma biopsies by Southern blot hybridization and determined gene dosage, as done above for *Mdm2*. Single allele or homozygous deletions were found in 1 (T16) and 4 (T1, T3, T11, and T36) cases, respectively (Fig. 4*B*). All occurred in

glioblastomas that expressed a wild-type *p53* and had no *Mdm2* abnormalities (Table 1), indicating a strictly mutually exclusive pattern of change among these three types of gene changes. Moreover, the cases with deleted *p16* sequences and, therefore, with normal *p53* gene sequences, exhibited little or no EGR-1 mRNA expression, with an average of 3.4%. Furthermore, these data suggest that reduced EGR-1 expression is a new molecular marker of astrocytic tumors without genetic alterations impairing the p53 pathway.

DISCUSSION

EGR-1 Is Suppressed in Astrocytic Tumors Compared with Normal Tissue. Here we have collected and evaluated 31 cases of fresh human astrocytomas or glioblastomas and have shown that EGR-1 expression is significantly decreased in tumor biopsies compared with a series of control tissues derived from normal brain, either human or rat. *EGR-1* gene expression in glioma specimens is decreased at both RNA and protein steady-state levels, and this finding is supported by the reduced number of *EGR-1*-positive cells found in the tumor specimens examined *in situ*. This phenomenon is common to both astrocytomas (WHO grade I-III) and glioblastoma. In the latter, EGR-1 mRNA down-regulation is less marked. However, even including these cases, the decreased expression of EGR-1 is still significant compared with normal controls. These observations on the expression of EGR-1 *in vivo*, therefore, support a number of previous studies of human tumor cell lines (11) including glioblastoma cell lines (11, 16), demonstrating that EGR-1 has significant tumor suppressor properties.

The ARF-Mdm2-p53 Pathway Plays a Critical Role in Glioblastomas. Previous studies of gliomas have shown that inactivation alterations of the ARF-Mdm2-p53 pathway commonly occur in a mutually exclusive pattern with a combined frequency ranging to >80% (25, 27, 28, 33, 39, 41). Indeed, we find that 13 cases exhibited either p53 mutations or Mdm2 amplification but not both. Five cases exhibited a deletion of *p16*, all with wild-type *p53* and *Mdm2* genotypes. Thus, the changes in the ARF-Mdm2-p53 pathway occurred in a strictly mutually exclusive pattern with a combined frequency of 58%. It is important to note that we examined the presence or absence of exon 2 of the *p16* gene, which does not exclude the possibility that a truncated ARF protein encoded by an intact exon 1 leading to functional exon 1α or exon 1β products may be present. However, there seems to be no evidence that deletion of exon 2 with expression of a functional truncated form occurs *in vivo*. Moreover, in those cases where p16INK4a and ARF deletion have been examined together *in vivo*, both are almost always deleted together (26–28, 39–41). Thus, although it is not possible to be unequivocal about the deletion of all forms of the ARF protein, our observations are entirely consistent with the conclusion that for gliomas, as for other major human neoplasms, disruption of ARF or p53 or overexpression of Mdm2 is a major mechanism of tumor progression.

EGR-1 Levels *in Vivo* Are High Only When *p53* Is Mutant. The major hypothesis of this study is that *EGR-1* is a tumor suppression factor and, therefore, would be inhibited in expression in fresh brain tumors, as inferred from glioblastoma cell line studies (16). Two major corollaries are that inhibition of expression of EGR-1 would occur regardless of the state of the ARF-Mdm2-p53 pathway if EGR-1 worked through a p53-independent mechanism (*i.e., EGR-1* mRNA absent in most cases), but that inhibition of expression would occur only exclusive of other changes in the ARF-Mdm2-p53 pathway if EGR-1 acted as an a member of the pathway (*i.e., EGR-1* mRNA absent only exclusive of other p53 pathway inactivating changes). Surprisingly both corollaries are manifested. *EGR-1* mRNA expression is reduced or absent in 87% of all cases, and the extent of reduction, to 15% of normal, was shown to be significant even when the average for all 31 tumor cases is compared with the normal value. This conclusion is supported by direct immunohistochemical analysis of protein expression. Thus significant inhibition of expression regardless of the state of the ARF-Mdm2-p53 pathway is observed. On the other hand, *EGR-1* mRNA expression is notably higher for the cluster of cases with *p53* mutations (average, 66% *versus* 19% and 3.4% for the cases with altered *Mdm2* and *p16* genes, respectively), and the values are much more variable, ranging to 200% of the normal value (Table 1). Indeed, the average level of expression for this group was shown to be significantly increased compared with all other cases. Thus, strong inhibition of expression of EGR-1 is exclusive of the group of cases with mutations in p53. Because most mutations observed here are thought to inactivate p53 (compare Table 1), this observation suggests that the mechanism of action of *EGR-1* is not entirely independent of that for inactivation of *p53* itself. Thus, during tumor progression, there appears to be reduced pressure to inhibit expression of *EGR-1* when *p53* has been inactivated. A model that provides an explanation of these effects is considered below.

What Is the Mechanism of Tumor Growth Suppression Operated by *EGR-1*? Much of the previous experimental evidence of the oncosuppressive activity of EGR-1 is based on the effects of constitutive reexpression of the gene in several tumor cell lines, including the glioblastoma U251 cell line (11, 16, 18), and by the suppression of expression of *EGR-1* in human tumor cell lines (13, 42). The reexpression of *EGR-1* leads to restoration of contact inhibition, morphological reversion, and the reduction in tumorigenicity (16). Most of these changes are referable to up-regulation of TGF-β1 because of direct transactivation of TGF-β1 by EGR-1. In fact, TGF-β1 is expressed and secreted in direct proportion to the level of expression of EGR-1 (16), and the effects of EGR-1 are reversed by the addition of anti-TGF-β1 antibodies (18). Indeed, TGF-β1 is recognized as a major growth regulator of naturally occurring gliomas, and inactivation of the TGF-β1 mechanism, especially by loss or mutation of the TGF-β1 receptor type II subunit, increases in frequency with the increasing grade of gliomas (19). This view is supported also by preliminary studies conducted *in vitro* by our group on an extended series of freshly explanted gliomas in which we observed a strong correlation between TGF-β1 secretion and *EGR-1* expression.

These observations suggest a model for the suppressor role of *EGR-1* and how this factor may be related to the p53 mechanism. Both p53 and the TGF-β1 signal transduction pathways regulate the same target gene, the cell-cycle regulator $p21^{Cip/1Waf1}$ (43, 44). Therefore, by directly transactivating the TGF-β1 promoter (18–42) and stimulating the secretion and subsequent activation of TGF-β1, EGR-1 may regulate

$p21^{Cip/1Waf1}$1 *via* an autocrine loop. Thus, EGR-1 itself may be a target of suppression during tumor progression, as for p53 and/or the TGF-β1 receptor. However, if p53 is the major regulator of $p21^{Cip/1Waf1}$ expression, tumor cells that first undergo complete inactivation of *p53* may be relatively insensitive to the inhibition of *EGR-1* expression. This mechanism provides an explanation for the bimodal distribution of *EGR-1* mRNA, *i.e.*, that relatively high mRNA values segregate with *p53* mutations (compare Table 1). In summary, we propose that *EGR-1* acts in human glioma as a tumor suppressor factor.

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