

Association between Functional *EGF*+61 Polymorphism and Glioma Risk

Bruno Marques Costa,¹ Paulo Ferreira,⁴ Sandra Costa,¹ Paulo Canedo,⁴ Pedro Oliveira,² Ana Silva,³ Fernando Pardal,³ Gianpaolo Suriano,⁴ José Carlos Machado,^{4,5} José Manuel Lopes,^{4,5} and Rui Manuel Reis¹

Abstract Purpose: Epidermal growth factor (EGF) plays a critical role in cancer. A polymorphism in the *EGF* gene (*EGF*+61) may influence its expression and contribute to cancer predisposition and aggressiveness. In the present study, we aimed to elucidate the role of *EGF*+61 in glioma susceptibility and prognosis.

Experimental Design: A case-control study involving 197 glioma patients and 570 controls was done. Univariate and multivariate logistic regression analyses were used to calculate odds ratio (OR) and 95% confidence intervals (95% CI). False-positive report probability was also assessed. The luciferase reporter gene assay was used to ascertain the functional consequences of this polymorphism.

Results: Corroborating the univariate analysis, the multivariate model showed that the G allele conferred higher risks for gliomas (OR, 1.32; 95% CI, 1.04-1.67), glioblastomas (OR, 1.47; 95% CI, 1.02-2.10), and oligodendrogliomas (OR, 1.55; 95% CI, 1.07-2.23). The GG genotypes were associated with increased risk for gliomas (OR, 1.71; 95% CI, 1.07-2.73), glioblastomas (OR, 2.03; 95% CI, 1.02-4.05), and oligodendrogliomas (OR, 2.72; 95% CI, 1.18-6.28). In addition, the AG+GG genotypes were associated with higher risk for gliomas (OR, 1.52; 95% CI, 1.03-2.23) and oligodendrogliomas (OR, 2.80; 95% CI, 1.35-5.79). No significant association was observed between the *EGF*+61 polymorphism and glioblastoma or oligodendroglioma patients' overall survival. The luciferase reporter gene assay exhibited a significant increased promoter activity for the G variant compared with the reference A allele.

Conclusions: These findings support the role of the *EGF*+61 polymorphism as a susceptibility factor for development of gliomas and show its implication on *EGF* promoter activity.

Central nervous system tumors represent only 2% of all cancer deaths; however, these tumors rank second as cause of cancer death in people below 39 years old (1). In fact, central nervous system tumor patients have the highest average years of life lost among all cancer types, with an average of 20.1 years (2). Gliomas are the most frequent central nervous system tumors, being astrocytic, oligodendroglial, and mixed gliomas (e.g.,

oligoastrocytic), the major histologic types. According to the WHO, gliomas can be classified into four grades of malignancy (3). Despite all advances in neurosurgery and adjuvant radiotherapy and chemotherapy, glioma patients' prognosis is dismal (4). Survival of glioblastoma patients, the most common and aggressive (WHO grade 4) form of glioma in adults, is very poor, with median survival times ranging from ~ 10 months in patients younger than 65 years to 3.5 months for those older than 65 years (5).

Deregulation of growth signaling pathways constitute a hallmark of cancer (6). Epidermal growth factor (*EGF*) encodes a ligand for the EGF receptor (EGFR). Upon EGFR activation, a cascade of intracellular signaling pathways is initiated that will ultimately influence cell proliferation, inhibition of apoptosis, and differentiation (6-9). Alterations of EGF/EGFR signaling occur frequently in gliomas by several mechanisms, including autocrine/paracrine stimulation loops, *EGFR* gene amplification, and activating mutations (e.g., EGFRvIII). These abnormalities are associated with glioma growth, invasion, and malignancy (3, 8, 10). Therefore, EGF plays a key role in the gliomagenesis (9, 10).

Besides somatic molecular alterations, a growing body of evidence has been demonstrating that common germ line polymorphisms may play a role in cancer risk and can influence tumor progression, prognosis, and therapies response (11-15).

Authors' Affiliations: ¹Life and Health Sciences Research Institute (ICVS), School of Health Sciences and ²Department of Production and Systems Engineering, University of Minho; ³Department of Pathology, Hospital S. Marcos, Braga, Portugal; ⁴IPATIMUP—Institute of Molecular Pathology and Immunology; and ⁵Medical Faculty, Department of Pathology, Hospital S. João, University of Porto, Porto, Portugal

Received 10/30/06; revised 2/3/07; accepted 2/15/07.

Grant support: Sixth Research Framework Programme of the European Union, Project INCA (LSHC-CT-2005-018704). B. Costa is a recipient of a fellowship grant from the Portuguese Ministry of Research (SFRH/BD/15258/2004).

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Requests for reprints: Rui Manuel Reis, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. Phone: 351-235-604825; Fax: 351-253-604820; E-mail: rreis@eceaude.uminho.pt.

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doi:10.1158/1078-0432.CCR-06-2606

Shahbazi et al. (16) identified a single-nucleotide polymorphism in the 5' untranslated region of the *EGF* gene (an A-to-G variant found 61 bp downstream of the *EGF* transcription start site—*EGF*+61 polymorphism). In this study, the authors found that the GG genotype was significantly associated with risk of malignant melanoma and that cells from individuals with the AA genotype produced significantly less EGF than cells from individuals with either the AG or GG genotypes. However, later independent studies have reported some conflicting results (17–22). Besides melanomas, Bhowmick et al. (23) showed a relationship between this functional polymorphism and the risk and aggressiveness of glioblastomas. By studying 42 American patients, they found that both frequencies of G allele and GA or GG genotypes were significantly higher in glioblastoma patients than in healthy individuals. Additionally, patients with the GA or GG genotype had a significant shorter progression-free survival than those with the AA genotype (23).

Considering the paramount role of EGF/EGFR signaling in gliomas and the potential implication of *EGF*+61 in glioblastoma development, we proposed to clarify the significance of this genetic polymorphism in the glioma susceptibility. We did a case-control study of 197 glioma patients and 570 cancer-free controls from Portugal. Associations of *EGF*+61 genotypes with tumor risk and patient prognosis were assessed. In addition, the functional consequences of the *EGF*+61 genetic variants were evaluated by luciferase reporter analysis.

Materials and Methods

Study population. In this case-control study, we enrolled 197 patients gliomas, diagnosed at Hospital S. João, Porto, and Hospital S. Marcos, Braga, Portugal. All new consecutive cases of glioma patients, submitted to craniotomy with available tumor material, were eligible, which represented ~60% of patient seen in the two hospitals during the period of January 1990 to December 2004. Tumors were classified according to WHO (3), and clinicopathologic features are summarized in Table 1. The control group was randomly selected from blood donors at Hospital S. Marcos, Braga, and it included 570 cancer-free individuals. All patients and controls were from Northwest Portugal and of Caucasian ethnic background. The procedures followed in the present study were in accordance with the institutional ethical stand-

ards. All the samples enrolled in the present study were unlinked and unidentified from their donors.

Genotyping. Cancer patients' genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor tissue, as previously described (24). No peripheral blood DNA was available from the majority of glioma patients. DNA from control individuals was extracted from leukocytes of blood samples by proteinase K/chloroform/isopropanol treatment (25). The purified DNA was used to determine the genotypes for the *EGF*+61 polymorphism, using PCR-RFLP methods as previously described (23). Briefly, the *EGF* region from nucleotide positions -78 to +164 was amplified by PCR, producing a 242-bp fragment. After restriction of the PCR products with 2 units of *AluI* (Fermentas) for 16 h at 37°C, 61G alleles were distinguished from 61A alleles by visualization of a single 193-bp fragment instead of 91 and 102 bp fragments on a 3% agarose gel stained with ethidium bromide.

Plasmid construction. To study the effect on the promoter activity of the *EGF*+61 A-G polymorphism, a 756-bp fragment between nucleotides -552 to +204 containing either the A (wild type) or the variant allele (G) was initially amplified from genomic DNA (isolated from subjects homozygous either for the A or G allele) with *EGF*-specific primers incorporating *MluI* and *HindIII* restriction sites (5'-ACGCGT-CATACTGTATCTCTTCATTGG-3' and 5'-AAGCTTTGGAAGCCAGTAA-GAAATACC-3', respectively). The two purified PCR products were subcloned into pCR2.1 vector (Invitrogen) to obtain plasmid pCR2.1-EGF+61A or pCR2.1-EGF+61G. The DNA sequences of inserts were verified by sequencing using M13 primers (Invitrogen). The two promoter sequences had no sequence differences other than the +61. The pCR2.1-EGF plasmids containing +61A or +61G were then digested with *MluI* and *HindIII* overnight at 37°C and subcloned into the pGL3-Basic vector (Promega Corporation), upstream of the firefly luciferase reporter gene, to generate pGL3-EGF+61A and pGL3-EGF+61G, respectively. All constructs were checked by direct sequencing using GLprimer2 and RVprimer3 (Promega) on an ABI PRISM 3100 Genetic Analyser sequencer (Applied Biosystems).

Cell culture, transfection, and luciferase assay. U251 glioma cells (kindly provided by Dr. Joseph Costello, University of California, San Francisco, San Francisco, CA) and MDA-MB-435 breast carcinoma-derived cells were grown in 50% DMEM and 50% Ham's F12 medium supplemented with 10% fetal bovine serum and 1% of penicillin and streptomycin (Life Technologies), in a humidified atmosphere at 37°C with 5% CO₂. Transient transfection experiments were done using the LipofectAMINE reagent (Invitrogen). Briefly, cells were seeded in DMEM at 5.0 × 10⁵ per well in six-well plates. Transfections were done the next day by mixing 1 µg of the pGL3 construct of interest and 0.5 µg of the control β-galactosidase-expressing vector, with LipofectAMINE 2000 (Invitrogen; LipofectAMINE/DNA ratio of 3:1) in 6 mL of serum-free and antibiotic-free Opti-MEM (Invitrogen) medium. Cells were incubated with 1 mL of the transfection mixture for 16 h at 37°C. Total cell extracts were prepared after 48 h incubation at 37°C in DMEM using 1× reporter lysis buffer (Promega), as described in the manufacturer's instruction manual. Twenty microliters of cell extract were mixed with 100 µL of luciferase assay reagent (Promega) to determine luciferase activity in a 1450 Microbeta luminescence counter (Wallac). The β-galactosidase activity was measured using 50 µL of cell extract. The luciferase activity of test plasmids is expressed as fold of induction of the test plasmid activity, after correction for transfection efficiency as measured by the β-galactosidase activity. Each assay was repeated in three independent experiments, including three replicates for the two constructs.

Statistical analysis. χ² and nonparametric Wilcoxon-Mann Whitney tests were used to compare the frequency distribution of age, sex, and *EGF*+61 genotypes and alleles between cases and controls. Moreover, the χ² test was used to verify that the observed allele distribution, in the control group, was in Hardy-Weinberg equilibrium. Odds ratios (OR) and 95% confidence intervals (95% CI) for the effect of *EGF*+61 variants on the risk for each glioma type were estimated by univariate and multivariate logistic regression analyses, adjusted for sex and age as

Table 1. Clinicopathologic features of gliomas and controls

Groups (WHO grade)	No. cases	Age, y (mean ± SD)	Male/female ratio
Controls	570	42.6 ± 0.6	0.76
Gliomas (1-4)	197	48.5 ± 1.2	1.24
Astrocytoma (1-4)	125	50.2 ± 1.5	1.16
Pilocytic astrocytoma (1)	9	25.1 ± 2.2	0.8
Diffuse astrocytoma (2)	28	36.6 ± 3.1	0.87
Anaplastic astrocytoma (3)	3	41.0 ± 8.5	3
Glioblastoma (4)	79	57.0 ± 1.3	1.32
Gliosarcoma (4)	6	63.8 ± 3.5	0.5
Oligodendroglioma (2-3)	66	46.0 ± 2.2	1.36
Oligodendroglioma (2)	33	41.2 ± 2.9	1.36
Anaplastic oligodendroglioma (3)	33	51.0 ± 3.0	1.36
Oligoastrocytoma	6		
Anaplastic oligoastrocytoma (3)	6	40.5 ± 5.4	2

Table 2. Univariate analysis of association between *EGF*+61 polymorphism and risk for each glioma group

<i>EGF</i> +61	Control	Glioma (1-4)	OR (95% CI)	Glioblastoma (4)	OR (95% CI)	Oligodendroglioma (2-3)	OR (95% CI)
Genotypes							
AA	173	44	—	19	—	9	—
AG	266	97	1.43 (0.96-2.15)	35	1.20 (0.66-2.16)	39	2.82 (1.33-5.96)
GG	131	56	1.68 (1.07-2.65)	25	1.74 (0.92-3.29)	18	2.64 (1.15-6.06)
AG+GG	397	153	1.52 (1.04-2.22)	60	1.38 (0.80-2.38)	57	2.76 (1.34-5.70)
Alleles							
A	0.537	0.470	—	0.462	—	0.432	—
G	0.463	0.530	1.31 (1.04-1.65)	0.538	1.35 (0.97-1.88)	0.568	1.53 (1.06-2.19)

NOTE: Bold-faced values indicate significant difference at the 5% level.

a continuous variable. False-positive report probability (FPRP) was calculated for observed significant associations accordingly to Wacholder et al (26). Following Wacholder et al.'s recommendation for rare tumors or initial studies, we have calculated FPRP for a range of prior probabilities from 10% to 0.1% and used a threshold of noteworthiness of FPRP ≤ 0.5 (26). Patient survival curves were assessed by the Kaplan-Meier method for glioblastoma and oligodendroglioma; the log-rank test was used to evaluate the differences. Statistical differences in luciferase reporter assays were assessed using a Student's *t* test. All statistical tests were two-sided, and significance was considered for $P < 0.05$. Data analysis was done using SPSS 14.0 software (SPSS, Inc.).

Results

***EGF*+61 and risk of glioma.** We analyzed 197 glioma patients and 570 cancer-free control individuals to study associations between *EGF*+61 polymorphism and susceptibility to different types of gliomas. The distribution of *EGF*+61 allele frequencies in the control group was in Hardy-Weinberg equilibrium ($P = 0.348$). A summary of clinicopathologic features of the controls and cases is shown in Table 1. The statistical analysis of age and sex distributions between control and case groups (gliomas, glioblastomas, and oligodendrogliomas) showed significant differences for all classes ($P < 0.05$).

When assessing the allele frequencies using univariate analysis (Table 2), we found that the G allele was associated with higher risk for glioma (OR, 1.31; 95% CI, 1.04-1.65) and oligodendrogliomas (OR, 1.53; 95% CI, 1.06-2.19). Using AA

genotype as reference, the OR analysis showed that both the GG and combined AG+GG genotypes were associated with increased risk for glioma (OR, 1.68; 95% CI, 1.07-2.65 for GG; OR, 1.52; 95% CI, 1.04-2.22 for AG+GG; Table 2). In oligodendroglioma cases, the AG, GG, and AG+GG genotypes were all associated with a higher risk (OR, 2.82; 95% CI, 1.33-5.96 for AG; OR, 2.64; 95% CI, 1.15-6.06 for GG; OR, 2.76; 95% CI, 1.34-5.70 for AG+GG; Table 2). In addition, we stratified gliomas in astrocytomas (grades 2 and 3, $n = 31$). A protective effect was observed for AG genotype (OR, 0.33; 95% CI, 0.13-0.82; data not shown).

We further used a multivariate logistic regression model adjusted for sex and age as a continuous variable (Table 3). The allelic analysis showed that the G allele was a risk factor for glioma (OR, 1.32; 95% CI, 1.04-1.67), oligodendroglial tumors (OR, 1.55; 95% CI, 1.07-2.23), and also glioblastoma (OR, 1.47; 95% CI, 1.02-2.10), when compared with the reference A variant. Consistent with the results obtained by the univariate analysis (Table 2), the GG and combined AG+GG genotypes conferred increased risk for glioma (OR, 1.71; 95% CI, 1.07-2.73 for GG; OR, 1.52; 95% CI, 1.03-2.23 for AG+GG; Table 3). In oligodendrogliomas, the AG, GG, and AG+GG genotypes were all associated with higher risks (OR, 2.83; 95% CI, 1.33-6.02 for AG; OR, 2.72; 95% CI, 1.18-6.28 for GG; OR, 2.80; 95% CI, 1.35-5.79 for AG+GG; Table 3). The GG genotype increased by ~2-fold the risk for glioblastoma (OR, 2.03; 95% CI, 1.02-4.05; Table 3) in this logistic regression model, whereas it failed to reach significance in the univariate analysis.

Table 3. Multivariate logistic regression analysis of the association between *EGF*+61 polymorphism and risk for each glioma group

<i>EGF</i> +61	Control	Glioma (1-4)	OR (95% CI)	Glioblastoma (4)	OR (95% CI)	Oligodendroglioma (2-3)	OR (95% CI)
Genotypes							
AA	173	44	—	19	—	9	—
AG	266	97	1.43 (0.94-2.15)	35	1.26 (0.67-2.3)	39	2.83 (1.33-6.02)
GG	131	56	1.71 (1.07-2.73)	25	2.03 (1.02-4.05)	18	2.72 (1.18-6.28)
AG+GG	397	153	1.52 (1.03-2.23)	60	1.50 (0.83-2.69)	57	2.80 (1.35-5.79)
Alleles							
A	0.537	0.470	—	0.462	—	0.432	—
G	0.463	0.530	1.32 (1.04-1.67)	0.538	1.47 (1.02-2.10)	0.568	1.55 (1.07-2.23)

NOTE: Bold-faced values indicate significant difference at the 5% level.

Table 4. False-positive report probability

EGF+61	OR (95% CI)	Power*	Reported P	Prior probability			
				0.1	0.05	0.01	0.001
Glioma (1-4)							
Genotypes (AA reference)							
GG	1.71 (1.07-2.73)	0.744	0.025	0.229	0.386	0.766	0.971
AG+GG	1.52 (1.03-2.23)	0.920	0.032	0.240	0.400	0.776	0.972
Alleles (A reference)							
G	1.32 (1.04-1.67)	1.00	0.021	0.157	0.282	0.672	0.954
Glioblastoma (4)							
Genotypes (AA reference)							
GG	2.03 (1.02-4.05)	0.483	0.045	0.453	0.636	0.901	0.989
Alleles (A reference)							
G	1.47 (1.02-2.10)	0.995	0.034	0.244	0.405	0.780	0.973
Oligodendroglioma (2-3)							
Genotypes (AA reference)							
AG	2.83 (1.33-6.02)	0.184	0.007	0.253	0.417	0.788	0.974
GG	2.72 (1.18-6.28)	0.236	0.019	0.422	0.606	0.889	0.988
AG+GG	2.80 (1.35-5.79)	0.182	0.005	0.213	0.364	0.749	0.968
Alleles (A reference)							
G	1.55 (1.07-2.23)	0.915	0.018	0.152	0.274	0.663	0.952

NOTE: Bold-faced values indicate the FPRP (≤ 0.5) for the most likely prior probability.
 *Estimation of statistical power to detect an OR of 2.0 with an α level equal to the observed P value.

The astrocytoma group (grades 2 and 3, $n = 31$), showed a protective effective for the AG genotype (OR, 0.32; 95% CI, 0.13-0.82; data not shown).

The calculation of FPRP showed that all of the above-mentioned associations remained noteworthy (FPRP ≤ 0.5) when a prior probability of association of $\geq 10\%$ was considered, and all except two associations (glioblastoma and oligodendroglioma with GG genotype) remained noteworthy when a prior probability of association of $\geq 5\%$ was considered (Table 4). Concerning the astrocytoma group (grades 2 and 3), the only remaining noteworthy association was the AG genotype when a prior probability of association of $\geq 10\%$ was considered (data not shown).

Survival and prognostic value of EGF+61. In glioblastoma ($n = 44$) and oligodendroglial tumors ($n = 45$) with available follow-up data, we also investigated the association between EGF+61 genotypes and overall survival time. Kaplan-Meier analysis did not show any statistically significant correlation between this polymorphism and glioblastoma patients' survival ($P = 0.365$). Similarly, no associations were found for oligodendroglial tumors ($P = 0.459$).

Functional role for the EGF+61 polymorphism by luciferase reporter assays. Having shown that the +61G allele of the EGF gene is associated with increased glioma risk, we sought to determine whether the EGF+61 A to G genetic variation has functional consequences. To accomplish this, we constructed two different pGL3-EGF+61 constructs for the +61A and +61G alleles (pGL3-EGF+61A and pGL3-EGF+61G, respectively) that were used to transiently transfect U251 glioma cells and MDA-MB-435 breast carcinoma-derived cells and assess their promoter activity. The average transcriptional activity of pGL3-EGF+61G was 1.65- and 1.53-fold higher than of pGL3-EGF+61A in U251 and MDA-MB-435 cancer cells, respectively, and these differences were statistically significant ($P = 0.0020$ and $P = 0.0099$, respectively; Fig. 1).

Discussion

Glioma carcinogenesis is a complex and still poorly understood process in which environmental and genetic factors cooperate with each other (27). With the exception of therapeutic ionizing radiation, no other environmental factors are clearly associated with brain tumors risk (28-31). Recently, several studies have focused on the etiologic role of germ line genetic polymorphisms in glioma development and prognosis (32). These studies included analysis of genes encoding proteins involved in DNA repair pathways (e.g., *MGMT*, *XRCC7*, and *ERCC2*; refs. 33-35), carcinogen metabolism (e.g., *GST* and *CYP2D6*; refs. 36-38), and the *EGF* gene (23).

In this case-control study, we investigated the association of the EGF+61 polymorphism with glioma susceptibility and

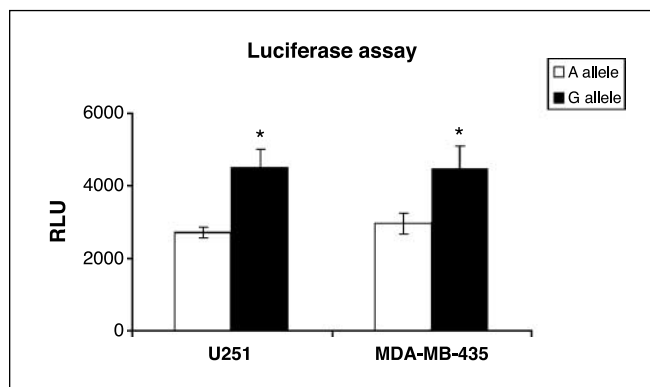


Fig. 1. Relative luciferase activity of human cancer cell lines U251 (glioma) and MDA-MB-435 (breast carcinoma) transiently transfected with a portion of the EGF promoter and 5'-untranslated region, harboring either A or G allele at the +61 locus. The results are representative of three independent experiments, including three replicates for both constructs. *, statistically significant difference using Student's t test ($P = 0.0020$ for U251 cells; $P = 0.0099$ for MDA-MB-435 cells).

patient prognosis in a large panel of Portuguese cases. It is well established that the distinct glioma subtypes arise from different genetic pathways (3, 39, 40); therefore, we have done a histologic stratification to elucidate more precisely the significance of the results. We found a significant association of the G variant with an increased risk of not only gliomas (OR, 1.35) but also glioblastomas (OR, 1.48) and oligodendroglial tumors (OR, 1.58). Notably, we showed that individuals with *EGF*+61 GG genotype have approximately a 2-fold increased risk for glioma and glioblastoma and approximately a 3-fold risk for oligodendroglial tumors. Our data is in agreement with a previous study reporting a significant association between the G allele and the risk of developing glioblastoma (23). The observed OR is likely to reflect a true association, as shown by the FPRP calculation. Bhowmick et al. (23) have observed a statistical association between the *EGF*+61 polymorphism and glioblastoma patients' progression-free survival. In the present series, no statistical association was found between the *EGF*+61 polymorphisms and glioblastoma and oligodendroglial tumors overall survival time. In the astrocytoma group (grades 2 and 3), we observed a protective effect associated with the AG genotype. This paradoxical association is most probably related to the rather small number of samples ($n = 31$) analyzed; however, supplementary studies are warranted to address this particular subtype of gliomas.

In the present work, the genotype analysis was evaluated in patient tumor tissue. Thus, we cannot exclude the existence of somatic alterations in the *EGF* locus (4q25), which would lead to misgenotyping. However, chromosomal abnormalities of the 4p25 region are infrequent in gliomas (3). Moreover, in 20 glioma cases with available peripheral blood DNA, genotyping of both blood and tissue DNA was done with 100% concordance.

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Acknowledgments

We thank Dr. J. Costello for kindly providing the glioma tumor cell line.

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