Genetic Alterations of Phosphoinositide 3-kinase Subunit Genes in Human Glioblastomas

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Genetic alterations of PI3K (phosphoinositide 3-kinase) subunits have been documented in a number of tumor types, with increased PI3K activity linked to gene amplification and mutation of catalytic subunits, as well as mutations of regulatory subunits. Among high grade gliomas, activation of the PI3K-AKT signaling pathway through loss of PTEN function is common. We therefore investigated whether genetic alteration of class IA PI3Ks might provide a mechanism for deregulation of this pathway in glioblastomas. We studied a series of glioblastomas with FISH to assess copy number of catalytic subunits (*PIK3CA* and *PIK3CD*) and with PCR-SSCP to screen for somatic mutations of conserved regions of both catalytic and regulatory subunits. FISH revealed frequent balanced copy number increases of both *PIK3CA* and *PIK3CD*, and one case showed an extra copy limited to *PIK3CA*. One glioblastoma exhibited a 9-bp deletion that encompassed the exon-intron junction of exon 12 of *PIK3R1*, documenting for the first time a mutation within a PI3K regulatory subunit in human glioblastoma. This deletion would be predicted to yield a truncated protein that lacks the inhibitory domain, resulting in increased PI3K activity. Furthermore, the case with selected *PIK3CA* copy number gain and the case with a truncating *PIK3R1* mutation both featured AKT activation without *PTEN* mutation. These results suggest that genetic alterations of class IA PI3K subunit genes can occasionally play a role in human glioblastoma by activating the PI3K-AKT signaling pathway independently of *PTEN* mutation.

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INTRODUCTION

Glioblastoma is the most common primary human brain tumor of adults. A variety of signal transduction pathways are deregulated in glioblastoma tumorigenesis. For example, amplification and/or overexpression of receptor tyrosine kinase (RTK) genes such as *EGFR* and *PDGFR* are common in glioblastoma, as is activation of RAS-MAPK and PI3K-AKT signaling pathways (27). Furthermore, RAS and AKT signaling pathways cooperate to induce glioblastoma formation in mouse models (6).

AKT activation is related to tumor progression (24), and various components of the AKT pathway are altered in many human cancers (25). In addition to RTK and PTEN alterations, Class IA PI3Ks are clearly involved in human oncogenesis. Class IA PI3K acts as a heterodimer comprised of a unique catalytic subunit (PIK3CA, PIK3CB or PIK3CD) and a regulatory subunit (PIK3R1, PIK3R2 or PIK3R3) (25). This complex phosphorylates PtdIns(4,5)P2 at the D3 position to form PtdIns (3,4,5)P3, which acts as a second messenger and leads to AKT activation. Through phosphorylation, activated AKT influences several downstream targets such as MDM2, BAD, NF-κB and mTOR, resulting in cellular growth, survival and proliferation (2, 25).

The regulation of the PI3K-AKT pathway by PTEN has provided evidence of the importance of this pathway in human glioblastoma (11). However, the *PTEN* gene is mutated in only 20% to 40% of glioblastomas (5, 11, 18, 22), whereas the AKT pathway is activated in over 80% of glioblastomas (6). We hypothesized that additional components of this pathway might be targeted by genetic changes in glioblastomas. Genetic alteration of PI3K subunits has been documented in a number of tumor types: increased PI3K activity has been linked to mutations within regulatory (17) and catalytic subunits (20), as well as gene amplification of catalytic subunits (13, 23, 26). We therefore investigated the different components of class IA PI3K genes to evaluate this hypothesis.

MATERIALS AND METHODS

Tissues and DNAs. Thirty cases of glioblastoma were obtained from Massachusetts General Hospital, Boston, Mass, after appropriate human studies approval. Tumor DNA was extracted from snap frozen tissues or formalin-fixed, paraffin-embedded tissues. Constitutional DNA was extracted from blood leukocytes with conventional methods (21). Based on a previous study of allelic loss of 10q25, we selected 8 cases with loss of heterozygosity (LOH), 10 cases that retained both alleles and 12 cases with unknown allelic status of 10q25 (19). Although chromosome 10 loss occurs in the great majority of glioblastomas, we biased our study toward cases having both alleles of 10q on the assumption that these cases would be less likely to have *PTEN* alterations and thus be more likely to harbor genetic changes of PI3Ks.

LOH analysis of PTEN region. To confirm LOH around the *PTEN* region, we performed LOH assays with 2 microsatellite markers at 10q23: *D10S2491* and *D10S583*; D10S2491 is a microsatellite marker within *PTEN* (1). We compared PCR products amplified from blood and tumor DNA, as previously reported (21).

Fluorescence in situ hybridization (FISH) analysis. Gene copy number of PI3K catalytic subunits (*PIK3CA* and *PIK3CD*) was investigated by FISH on touch prepara-

tions because we were concerned about the exact assessment of single copy number gains in tissue sections, given that we might have encountered low copy number increases which would have been more difficult to evaluate on tissue sections with their "cut" nuclei. We reasoned that touch preparations, with intact nuclei, would give a more exact assessment of single copy number gains. We therefore only studied 10 cases for which frozen tissue was available. Touch preparations were made and fixed in methanol/acetic acid. We obtained BAC clones for each catalytic subunit, according to the UCSC database: RP11-245C23 (*PIK3CAI*)/ RP11-115G3 (3p control); and RP11-558F24 (*PIK3CD*)/ RP11-459O3 (1q control). Each probe was confirmed by mapping on metaphase control slides and PCR with specific primers. FISH was performed as described previously, with some modification (16). In brief, BAC DNA probes were labeled with Cy3 and FITC by nick translation. Hybridization was followed by washing and counterstaining with 4,5-diamidino-2-phenylindol (DAPI). More than 100 cells were counted for each case and probe, and the average used to calculate copy number.

Single strand conformation polymorphism (SSCP) analysis and direct sequencing. We designed primers for C-terminal conserved domains encoded by 5 exons of the regulatory subunits, and for the p85-binding domain and kinase core domain of the catalytic subunits: exon 11-15 of *PIK3R1* and *PIK3R2*, exon 6-10 of *PIK3R3*, exon 1, 15-19 of *PIK3CA*, exon1, 2, 16-20 of *PIK3CB* and exon 2, 3, 16 to 20 of *PIK3CD*. The primer sequences and PCR conditions are summarized in Table 1. *PTEN* mutation status was evaluated as described previously (21). Tumor specific shifts on SSCP were evaluated by direct sequencing with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation, Cleveland, Ohio).

Immunohistochemistry. We studied 20 glioblastomas, for which formalin-fixed, paraffin-embedded tissues were available for immunohistochemistry using a conventional avidin-biotin complex method. For evaluating AKT activation, we used an antibody specific for phospho-AKT (Ser 473) (Cell Signaling Technology Inc., Beverly, Mass); after antigen retrieval for 15 minutes in a microwave, each slide was incubated with 10% normal goat serum (Vector Laboratories Ltd., Burlingame, Calif) followed by overnight incubation at 4°C with a 1:100 dilution of p-AKT antibody. For evaluating wild type EGFR expression, we used the EGFR.113 antibody (Novocastra

Table 1A. Primer sequences for screening of PI3K subunit genes (regulatory subunits)				
Exon	**size**	**Tm**	**Upper primer**	**Lower primer**
PIK3R1 (p85α)				
exon11	233	48	TTT TTA AAA TTA TGT TGC AGG	CTT TAA ATG GTT TTA GAG GA
exon12	249	42	ATA ATA ACA AAT ACG TTT C	CAC CAA TTA TTC ATG TAT AG
exon13	140	46	AGT TAA TGC GTT CTC TTT TC	AAT CTT CTG CTA TCA CCA TC
exon 14	229	50	TGC ACT CTT CAT TTA GAA AC	TTT GCT GGA GAT ACA TAC AC
exon 15	234	52	TCT TCT CTC CTC TCT AGG GT	CAG GAG AAG GAT CAA AGA GT
PIK3R2 (p85β)				
exon11	239	62	CTG AGC TGC GCC CCC TCC TCC	GCT TCC TTC CCT CCC TGG CTT G
exon12	242	64	GCC ACC CCA CCC CTC CCA CA	GCA GTC GCC AGG GAG CCG CA
exon13	135	58	CTG CAC AAG CCC ACC TTT CC	CTC CCT GCC TCC CTC CAG AC
exon14	190	62	CCC ACC CCA GCC AGT ACG CA	TCC ACT CAC ACC ACG GAG CA
exon15	167	66	TCC CCC TCT CGT CTG CCC CC	GCG CGT CGT TGT GCT GCA CC
PIK3R3 (p55γ)				
exon6	213	48	AAT AAA GAT AAT GAA GCC TG	CCT TGG AGG TAC TAA GTA GA
exon7	233	48	TTT CTA TTT TAG CCT TAA CA	CAC TCC CAG ACT ACT TAC AC
exon8	146	48	AGA GTT TGC ATG GAT ACT GA	CTG ACA GAA AGG TAT AGA GA
exon9	202	44	TAA TAT TTA ATT TTT AGG AAC	CAG AGA AGA CTT ACA CCA C
exon10	241	52	CAT TTC TTC TGT GGT TTG TC	CTT CCC ACT TCC TCT TTA TC
Table 1B: Primer sequences for screening of PI3K subunit genes (catalytic subunits)				
Exon	**size**	**Tm**	**Upper primer**	**Lower primer**
PIK3CA (p110α)				
exon1-1	221	46	TTA TAT GTA AAA CTT GCA AAG A	CTT GAA GAA GTT GAT GGA GG
exon1-2	231	50	ATA CCC CCT CCA TCA ACT TC	GCT TTA TGG TTA TTT GCA TTT T
exon15	220	46	GTT TTT AAC TAT TTT AAA GG	TTT CAT GGT TCA TAA ATA TC
exon16	202	46	AGC TGT ATT TGT TTT TCA TTT	TAC AGG TTG CCT TAC TGG TT
exon17	236	46	TTT AAT TGT AAA CGT GTT ACT	GAT TAA CAT AGG AAG AAA GA
exon18	224	46	GTT TAT TCT TTG TAG ATA TG	AGA AAT TAT GTT ATA GTT TG
exon19	201	46	TTT CTT ATT TTT GAA AGC TG	TTT ATT TTG TGT TTT TAA TTG
PIK3CB (p110β)				
exon1	175	50	GGC GGT GGA TTC ACA GAT AG	ATT TCC CCC AAG TGA CAC AG
exon2	268	36	ATG TCT TAT TAT TTT TTC TC	TTT TAT TTT TTA ATT TAC CT
exon16	170	46	GTT GAC TAA TTT TCC CCA CT	GAA ATT TGA TGT TAA AAA TCT
exon17	169	46	CTT GTA ATG AAG CAG TTT GT	CTA GAT ATT TTT GCT ATG GG
exon18	224	46	GAA AAT ATG ACT CTA TGC CC	ATT AAA CAG AAT AAA CTA ACC
exon19	241	54	TGC TTC TGT TCT TTT CTC TTG	AAA AGA ATC TGG CTG AGT TG
exon20	231	46	GGA AAT TAA ATC CCA TTT GT	CCT GGT CAG ATA AGA AAA TGT
PIK3CD (p110δ)				
exon2	246	50	ATT TTG CCA TTT CTT CAT TT	TCT CTC TCT CTC TGA GCA CC
exon3	263	60	GGT GGA GGG GCT GAC CGG TG	CCT TTG CCG ATG AGG AGG CTG
exon16	204	60	ACC CCT GGG AGG CCG GTA GA	GTA CAG GCG GGG TGT GGG GA
exon17	157	60	AAC TCA CGC TTC TCC TCC CA	GGG CAC AGA CAC CAA GGG AC
exon18	218	60	GTC CCT CTT CCC CCT TGC CT	GGA ACC TAT CCC TGG GCC TG
exon19	167	60	TGC TTT TTA ATC TTC CCC ACC C	CCA GCA CCA GCC CCT GTA CC
exon20	201	58	TTC TCA ATC CTC CCC CTC CTC	TGG GGC TCA GGC ACT CTC AC

Table 1. Primers for SSCP analysis (see text).

	SSCP	FISH PIK3CA (control)	FISH PIK3CD (control)	IHC pAKT	PCR EGFR	IHC wt EGFR	IHC EGFR vIII	LOH 10q
Case								
1650	*PIK3R1* ex12	2 (2)	2 (2)	+	amp	+	-	LOH
1504	-	4 (3)	3 (3)	+	amp	+	-	-
8054	*PTEN* ex8	2 (2)	2 (2)	+	-	-	+	-
1532	*PTEN* ex7	2 (2)	2 (2)	+	amp	+	-	LOH
1524	-	3 (3)	3 (3)	+	amp	+	-	LOH
1690	-	3 (3)	3 (3)	nd	-	-	-	ni
7054	-	3 (3)	2 (2)	nd	-	nd	nd	-
1518	-	3 (3)	3 (3)	-	-	-	-	-
1640	-	2 (2)	1 (2)	-	amp	+	-	LOH
1514	-	2 (2)	2 (2)	-	-	-	-	-
1520	*PTEN* int3	nd	nd	+	amp	-	-	LOH
1688	*PTEN* ex7	nd	nd	nd	amp	nd	nd	LOH
1642	-	nd	nd	+	amp	+	-	-
1646	-	nd	nd	+	-	+	+	-
1644	-	nd	nd	+	amp	+	-	-
1808	-	nd	nd	+	-	+	-	-
1654	-	nd	nd	+	amp	-	+	-
1664	-	nd	nd	-	amp	-	-	-
1672	-	nd	nd	+	-	-	+	LOH
1682	-	nd	nd	+	-	-	+	-
1656	-	nd	nd	+	-	-	-	-
1660	-	nd	nd	+	-	-	+	-
1668	-	nd	nd	+	-	-	+	LOH
1680	-	nd	nd	nd	amp	nd	nd	LOH
1530	-	nd	nd	nd	-	nd	nd	-
1534	-	nd	nd	nd	-	nd	nd	LOH
1578	-	nd	nd	nd	-	nd	nd	-
1638	-	nd	nd	nd	amp	nd	nd	-
1814	-	nd	nd	nd	-	nd	nd	-
6360	-	nd	nd	nd	-	nd	nd	-

Table 2. Summary of results. SSCP = single strand conformational polymorphism analysis; *PIK3R1* and *PTEN* designate cases with mutation of the respective genes, with exonic (ex) and intronic (int) location of mutations indicated. For the FISH results, the copy number of *PIK3CA* or *PIK3CD* are given, with copy number of the corresponding control arm following in parentheses. IHC = immunohistochemistry; (+) indicates positive staining (see Methods). PCR EGFR = differential PCR for EGFR gene amplification; amp indicates gene amplification. wt = wild-type. LOH = loss of heterozygosity; ni = non-informative at all studied markers. For each assay, (-) represents a normal result, whereas nd indicates that the assays was not performed on that case.

Laboratories Ltd., Newcastle, United Kingdom); after antigen retrieval for 10 minutes in a microwave, each slide was incubated with 10% normal goat serum followed by overnight incubation at 4°C with a 1:30 dilution of the EGFR antibody. For evaluating EGFR vIII expression, we used the vIII mutant-specific antibody (Zymed Laboratories, Inc., South San Francisco, Calif); after antigen retrieval for 15 minutes in a microwave, each slide was incubated with 10% normal goat serum followed by overnight incubation at 4°C with a 1:250 dilution of the EGFRvIII antibody. For all assays, slides were then incubated with a biotinylated secondary antibody (Vector) for 30 minutes, and the avidin-biotin complex (Vectastain ABC kit, Vector) for 30 minutes. Diaminobenzidine tetrahydrochloride was used to visualize specific antibody. The slides were counterstained with Harris hematoxylin. When more than 20% cells showed moderate or strong staining, the tumor was considered positive.

Multiplex PCR for EGFR amplification. *EGFR* amplification was examined using a comparative multiplex PCR assay (12, 15).

RESULTS

The results are catalogued in Table 2. Of the 30 glioblastomas, 29 (97%) were informative for 10q markers and 10 (34%) had allelic loss of the *PTEN* region. Of the 30 glioblastomas, 13 (43%) showed *EGFR* amplification, and 4 (13%) had *PTEN* mutations. The *PTEN* mutations included a 34-bp insertion in exon 7, a 4-bp deletion in exon 8, a 1-bp insertion in exon 7 and a point mutation in intron 3. All of the coding region mutations create premature stop codons via frame shifts, predicting truncated proteins. (The frequency of PTEN mutation in our series is lower than previ-

ous reports, because of our sample bias; see Methods.) Sixteen (80%) cases of the 20 cases available for immunohistochemistry were positive for phospho-AKT.

Of the 10 glioblastomas studied for *PIK3CA* and *PIK3CD* amplification using FISH, 4 cases had trisomy of both chromosomes 1 and 3, suggesting the possibility of triploidy. However, one case had an extra copy of only the *PIK3CA* gene (3q26.3) (Figure 1). This case had AKT activation, as shown by positive immunohistochemistry with the phospho-specific AKT antibody, but no *PTEN* mutation.

One case demonstrated a tumor-specific shift in the *PIK3R1* gene on SSCP analysis (ie, the shift was not present in the sample of blood DNA). Direct sequencing revealed this to be a 9-bp deletion in the exon-intron junction of exon 12 (Figure 2A). This deletion would prevent proper splicing of intron 12 and would create a read-through transcript. The sequence of this new transcript encodes a premature stop codon at the end of exon 12 (Figure 2B), which would be predicted to give rise to a C-terminal deletion (Figure 2C). This case had activated AKT on immunohistochemistry and no *PTEN* mutation.

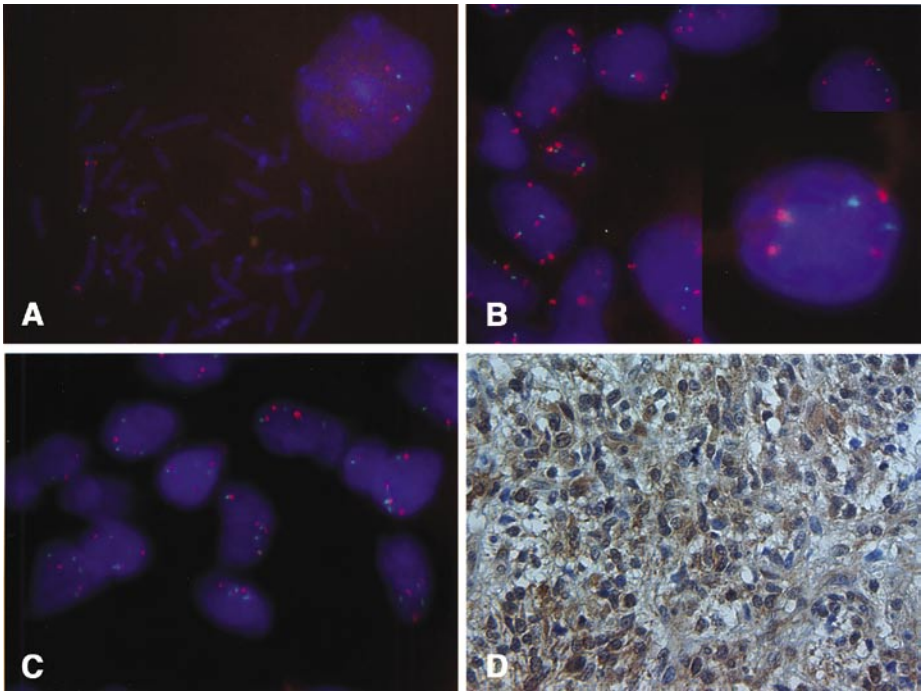

Figure 1. *FISH and immunohistochemistry results on GBM1504.* **A.** FISH of metaphase spread of normal control cell showing specific hybridization of RP11-245C23 (*PIK3CA*) at 3q26 (red signals) and RP11-115G3 (control) at 3p25 (green signals). **B.** GBM1504 with 4 copies of *PIK3CA* (red) and three control 3p signals (green), consistent with an extra copy of PIK3CA in addition to trisomy of chromosome 3. **C.** GBM 1504 with an average of 3 signals for the *PIK3CD* gene (red) and the control locus on 1q (green). **D.** pAKT immunohistochemistry shows diffuse cytoplasmic positivity in GBM1504.

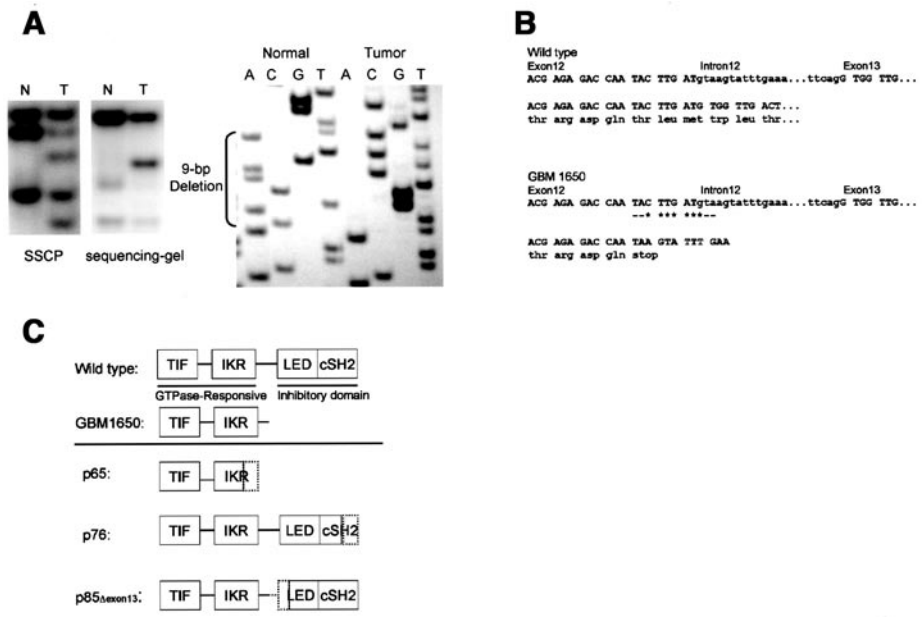

Figure 2. *Mutation in the PIK3R1 regulatory subunit gene.* **A.** GBM1650 displayed a tumor (T) specific shift on SSCP analysis (when compared with blood DNA, designated N), and a tumor specific, shorter allele and a retained normal allele when separated on a denaturing, sequencing gel. Direct sequencing revealed this alteration to be a 9-bp deletion in the exon-intron junction of exon 12 (right panel). **B.** Genomic, cDNA and amino acid sequences involved in the deletion. The deletion would prevent proper splicing of intron 12 and would create a read-through transcript, encoding a premature stop at the end of exon 12. **C.** The stop codon is located just between the GTPase responsive domain and inhibitory domain. Oncogenic truncated forms of p85α also lack a part of the inhibitory domain (dotted regions; see references 8, 9 and 17).

DISCUSSION

Phosphoinositide 3-kinase (PI3K) is a key component of RTK signaling, with PI3K leading to activation of AKT via phosphorylation. In human glioblastomas, AKT activation has been reported in over 80% of tumors (6), whereas the frequency of *PTEN* gene alteration is only 20% to 40% (5, 11, 18, 22), suggesting that other components of this pathway could activate AKT in tumors with an intact *PTEN* gene. The EGFRvIII mutant apparently activates the AKT pathway in the absence of *PTEN* alterations (4) and PI3K-AKT signaling is activated constitutively in EGFRvIII transformed cells (14). On the other hand, genetic alteration of other components of this pathway, such as *IRS2* and *PIK3CB*, are found in only small fraction of glioblastomas, and neither *PIK3CA* nor *PIK3CD* amplification has been detected (10).

We focused on class IA PI3Ks since these have been implicated in human oncogenesis. Amplification of *PIK3CA* (p110α) and mutation of *PIK3R1* (p85α) have been reported in wide spectrum of human cancers (13, 17, 23, 25, 26). *PIK3CA* copy number gains have been detected in 64% of human glioblastomas using array-based comparative genomic hybridization (7), but no amplification of *PIK3CA* could be detected using duplex-PCR assays (10). To resolve these issues, we used FISH,

which is both sensitive and quantitative, to evaluate copy number of *PIK3CA* and *PIK3CD*. Although a number of cases had balanced increases of both *PIK3CA* and *PIK3CD*, one case showed selective gain of *PIK3CA*. Low level increase in *PIK3CA* copy number has been reported in other human cancers, with the low level copy number increases affecting expression of p110α and leading to higher lipid kinase activities, even when 3 balanced copies of *PIK3CA* were detected (23). Given these data, extra copies of *PIK3CA* could lead to AKT activation. Indeed, we detected AKT activation on immunohistochemistry in the case with one extra copy of *PIK3CA*. Given the absence of *PTEN* mutation in this case, it is tempting to speculate that the extra *PIK3CA* gene could play a role in AKT activation. Of note, since the original submission of this manuscript, Samuels et al have reported mutations in the *PIK3CA* gene in a variety of human cancers, including some glioblastomas (20).

Mutation of *PIK3R1* has also been demonstrated in other types of cancer. The inter-SH2 region plays an important role in regulating the function of the lipid kinase. C-terminally truncated forms of this regulatory subunit can function as an oncogene. The GTPase-responsive domain and the inhibitory domain are found in the inter-SH2 region, and form a molecular switch. The inhibitory region includes the LED and C-terminal SH2 domains. Deletion of either domain abolishes the inhibitory function of the PI3K regulatory subunit, resulting in constitutive activation of PI3K-AKT signaling (3). All reported oncogenic truncated forms of PIK3R1, such as *p65* and *p76*, lack part of the inhibitory domain (8, 9). The deletions of exon 13 in primary human ovarian and colon cancers also lack part of the LED domain (Figure 2C) (17). These oncogenic truncated forms support the idea that a defect of the inhibitory domain in the presence of a retained GTPase-responsive domain could activate AKT. Our case with a 9-bp deletion in the exon-intron junction of exon 12 predicts a premature stop codon at the end of exon 12 located just between the GTPase responsive domain and the inhibitory domain. It seems highly likely that this truncated form would activate AKT, since the entire inhibitory domain would be lost whereas the GTPase-responsive domain would remain intact.

AKT activation was present in both cases with genetic alterations of PI3K. These 2 cases had neither *PTEN* mutation nor EGFRvIII expression (Table 2). Therefore, although uncommon, genetic alteration of *PI3K* subunit genes occurs in human glioblastoma and may represent a means of activating AKT signaling that is independent of *PTEN* mutation. The recent report of mutations in the *PIK3CA* gene in a variety of human cancers, including some glioblastomas (20), further supports involvement of these genes in human glial tumorigenesis.

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