REVIEW ARTICLE

Novel *PLA2G4C* Polymorphism as a Molecular Diagnostic Assay for 19q Loss in Human Gliomas

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***PLA2G4C*, encoding cytosolic phospholipase A_2-γ (cPLA$_2$-γ), is a 17-exon gene located on chromosome 19q13.3 within the putative glioma tumor suppressor gene region. Given the clinical importance of assessing 1p and 19q loss in human gliomas, the development of convenient and practical assays for detecting allelic loss is of considerable priority in neuro-oncology. We report a minisatellite polymorphism in the untranslated region of exon 1, with allelic variants that have one, two or three 27-bp repeats. The polymorphism is informative in 55.7% of a reference population, and accurately detects allelic loss of 19q in human gliomas. This novel marker offers distinct advantages for assessing 19q status in malignant gliomas. The relatively large size of the repeats allows detection of allelic variants with standard ethidium bromide-stained agarose gels and the *PLA2G4C* marker is the closest polymorphism to the smallest common deletion area in the putative glioma tumor suppressor gene region. These characteristics suggest that the *PLA2G4C* polymorphism will be a convenient and practical assay for clinical and research evaluation of 19q status in human gliomas.**

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Introduction

Of the various genetic alterations described in the diffuse gliomas, chromosome 19q loss is of particular interest for a number of reasons. Loss of 19q is the only genetic alteration that occurs commonly in all three major types of diffuse gliomas (astrocytoma, oligodendroglioma, and oligoastrocytoma) (7). Loss of 19q, in combination with 1p loss, is also associated with specific clinical features in oligodendroglial tumors. Those anaplastic oligodendrogliomas with 1p and 19q loss are more often chemosensitive (2) and demonstrate more durable responses to chemotherapy (5). In addition, patients whose oligodendrogliomas and anaplastic oligodendrogliomas have 1p and 19q loss have longer overall survival from the time of diagnosis (2, 5, 15). These observations suggest that the long arm of chromosome 19 harbours a tumor suppressor gene that, when inactivated, contributes to glioma tumorigenesis and confers clinically relevant phenotypes.

Given the clinical importance of assessing 1p and 19q loss in oligodendrogliomas and the biological importance of identifying the 19q glioma gene, much attention has been directed toward clinical assays for 1p and 19q loss (2, 8, 14), as well as research approaches for mapping the 19q locus (12, 16, 19). Two major approaches have been taken: loss of heterozygosity (LOH) assays with microsatellite markers and fluorescent in situ hybridization (FISH). LOH assays capitalize on small, highly informative polymorphisms that can be amplified by polymerase chain reaction (PCR) and separated on sequencing gels. Such techniques offer the advantage of easy interpretation of results, but suffer from the need for DNA extraction, polyacrylamide gels and, in most laboratories, the dependence on radionuclide labeling of DNA. FISH, on the other hand, is performed directly on tissue slides, obviating the need for DNA extraction, PCR, gels and radioactivity, but is a more problematic assay to establish and to interpret, since hybridizations may be difficult and quantification of signals is often time-consuming. Other techniques may improve the ability to detect allelic losses in gliomas, but are not likely to be widely used in the near future, either because of expense or difficulty with use. For instance, silver staining can substitute for radioactivity in LOH assays (18) but is cumbersome to employ, and automated approaches that employ fluorescent labeling or quantitative PCR rely on expensive equipment that is not widely available (8).

There is therefore a need for improved methods to detect allelic losses of 1p and 19q. We report a polymorphism in the *PLA2G4C* gene at the glioma candidate region on 19q that is informative and that can be assayed using simple agarose gels without radioactive labeling. This polymorphism could prove useful for simple molecular diagnostic clinical testing, as well as for map-

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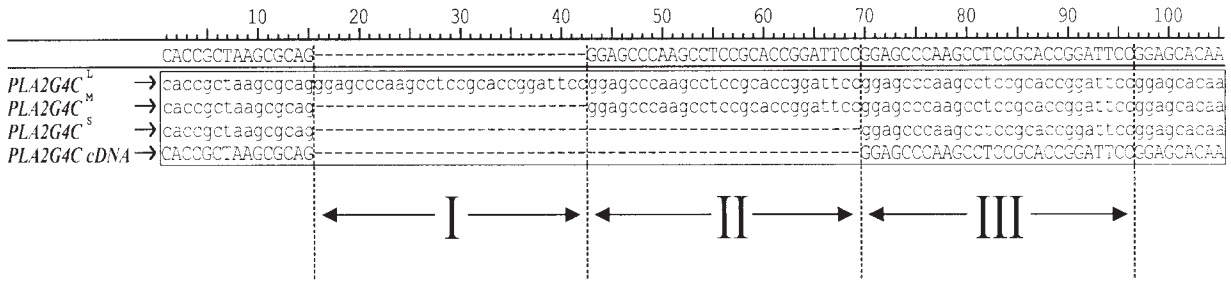

Figure 1. Repeated 27-bp motif in exon 1 of *PLA2G4C*: *PLA2G4C*^{S} has one, *PLA2G4C*^{M} has 2, and *PLA2G4C*^{L} has 3 copies. The published cDNA sequence *PLA2G4C* is included (GenBank accession number NM_003706).

ping and evaluation of genes in the 19q glioma candidate region.

Material and Methods

Computer-based analysis was performed with SeqMan II and EditSeq II (DNAstar, Madison, WI). GenBank files AF065214, AF058921, and NM_003706 were retrieved, and the sequence assembled using the *PLA2G4C* UniGene cluster Hs.18858. Blast and Sequencher 4.0 (Gene Codes Corporation, Ann Arbor, MI) were used to determine the number of exons and the exact location of exon-intron borders from HTGS (High Throughput Genome Sequence) database entries. For amplification of the second half of *PLA2G4C* exon 1 (*PLA2G4C*^{E1P2}), 2 oligonucleotides, 5′-CGCGG AGATT CCGGC TGAAG-3′ and 5′-TATGG CCGCC CCAGC GGGAT-3′, were used (32 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds), yielding a PCR product spanning the exon-intron border. Based on the available sequence of *PLA2G4C*, a 179-bp product was expected. The PCR products were detected on 2% agarose gels stained with ethidium bromide. The same primers were used for SSCP and sequencing studies. PCR products were sequenced directly using the fmol DNA Cycle Sequencing System (Promega, Madison, WI) and resolved using SequaGel-6 (National Diagnostics, Atlanta, GA) gels. For SSCP analysis, PCR products were separated on 8% non-denaturing polyacrylamide gels with 10% glycerol for 20 to 24 hours at 8 W, and visualized autoradiographically. All cases were screened for LOH with at least three different microsatellite markers (D19S219, D19S112, and D19S412), and in some cases with D19S596 as well.

DNA was extracted from blood samples of 41 glioma patients and 5 normal controls. To determine the normal allelic distribution, DNA was also obtained from an unrelated subset of the Centre d'Etude du Polymorphisme Humain, Paris (CEPH) reference panel. Tumor DNA was extracted from microdissected, formalin-fixed, paraffin-embedded sections or fresh, frozen biopsy samples of 41 gliomas, and from 13 glioblastoma cell lines. The primary gliomas comprised 18 glioblastomas, 4 anaplastic oligodendrogliomas, 1 anaplastic oligoastrocytoma, and 18 grade II oligodendrogliomas.

Results

Upon amplification of genomic DNA, 3 different allele sizes of *PLA2G4C*^{E1P2} were detected on agarose gels: 179-bp *PLA2G4C*^{S}, 206-bp *PLA2G4C*^{M}, and 233-bp *PLA2G4C*^{L}. No other exon of *PLA2G4C* showed sizes other than those expected (data not shown). Sequencing of the *PLA2G4C*^{E1P2} alleles demonstrated that *PLA2G4C*^{S}, *PLA2G4C*^{M}, and *PLA2G4C*^{L} had 1, 2, and 3 copies of a 27-bp motif (5′-GGAGC CCAAG CCTCC GCACC GGATT CC-3′), respectively, with the repeats located in the 5′-untranslated region of the gene (Figure 1). Based on the GenBank cDNA entry NM_003706, the original motif aligns between positions 206 and 232 (*PLA2G4C*^{S}). Available cDNAs, hESTs and genomic sequences related to *PLA2G4C* were assembled and analyzed. The hESTs from UniGene cluster Hs.18858 were compared to the GenBank entries NM_003706, AF065214, AF058921, XM_009119 and AK022440. One of 8 hESTs and the mRNA GenBank entry XM_009119 had 2 copies of the repeat (*PLA2G4C* M). Searching for *PLA2G4C* E1P2 within genomic sequences yielded one copy of the repeat sequence in the publicly available draft version of chromosome 19 (GenBank NT_011190), while 2 copies were found in LLNL BAC BC694629 (GenBank AC010458). None of the publicly available expressed or genomic sequences had 3 copies (*PLA2G4C* L).

To determine the informativeness of this polymorphism, allele frequencies were determined in 46 unrelated individuals. Of these, 28 (61%) showed a heterozygous pattern, while 18 (39%) were homozygous. The frequencies of the 3 alleles were 51.1% (*PLA2G4C*^{S}), 34.8% (*PLA2G4C*^{M}), and 14.1% (*PLA2G4C*^{L}). The

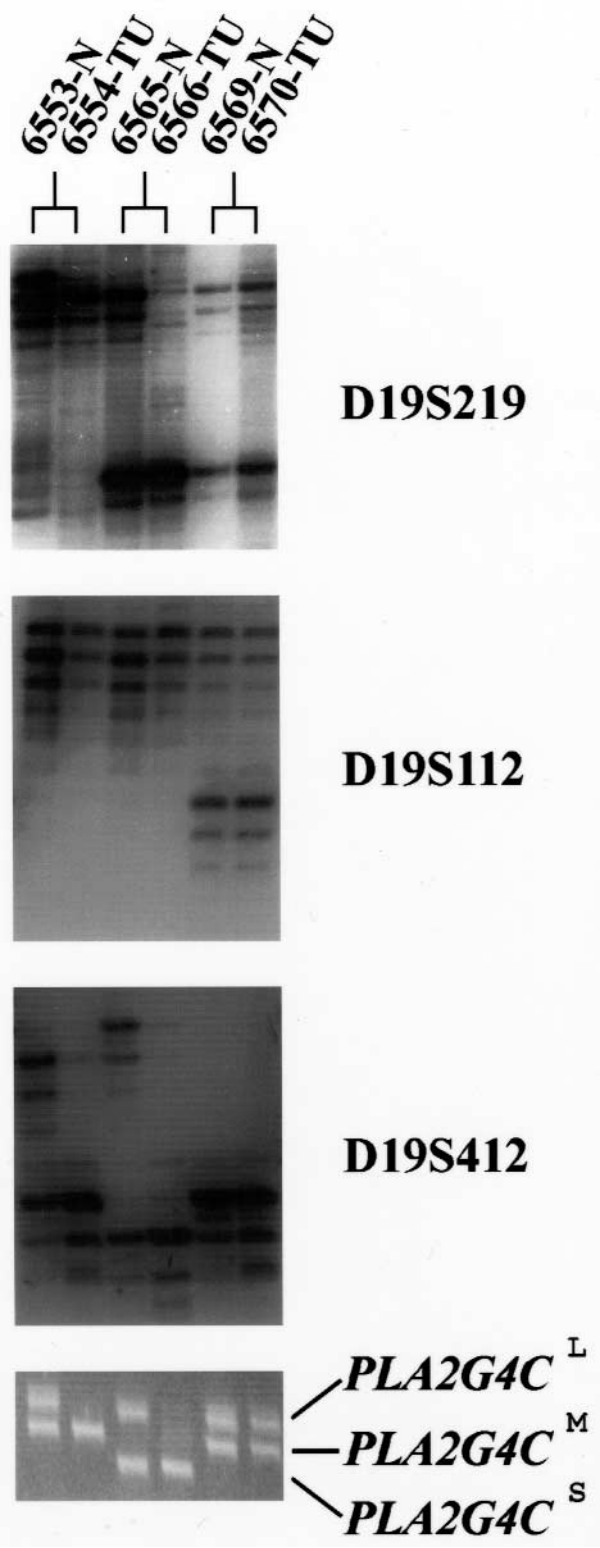

Figure 2. Comparison of LOH results from three microsatellite markers (D19S219, D19S112, D19S412) and the *PLA2G4C*^{E1P2} assay; *PLA2G4C*^{S} = 179 bp, *PLA2G4C*^{M} = 206 bp, and *PLA2G4C*^{L} = 233 bp; odd numbers represent bloods, while even are tumors. Cases 6553/6554 and 6565/6566 show loss of one 19q allele in the tumor, while case 6569/6570 retains both alleles.

genotype of 70 unrelated CEPH references was also determined. Of these, 39 (55.7%) were informative. Within this group, the allele frequencies of *PLA2G4C*^{S}, *PLA2G4C*^{M}, and *PLA2G4C*^{L} were determined to be 53.6%, 40%, and 6.4%, respectively. In combination, 67 (57.8%) of 116 were informative. Additionally, the cPLA2-γ^{E1P2} status of 13 previously genotyped glioblastoma-derived cell lines was tested. As expected, the three cell lines with known allelic loss had only a single band at the *PLA2G4C*^{E1P2} polymorphism. Of the remaining 10 cell lines known to have two 19q alleles, 7 were informative for *PLA2G4C*^{E1P2} and demonstrated 2 bands; the other 3 had only one band, indicating that these patients were homozygous for the polymorphism or that these cell lines had localized interstitial deletions affecting *PLA2G4C*.

The ability for this marker to detect allelic loss was then tested in 41 blood-tumor DNA pairs that had been studied and found to be informative with the conventional microsatellite markers using radionuclide labeling and polyacrylamide sequencing gels. Of the 24 cases that were informative at *PLA2G4CE1P2*, 9 had LOH and 15 retained both alleles; these results were entirely concordant with the microsatellite LOH data.

Discussion

A set of microsatellite markers has been established to analyze the allelic status of chromosome 19q. Commonly used markers include D19S219, D19S112, D19S412, D19S241E, D19S606, D19S596, and D19S246 (1, 6, 10, 19). Multiple studies have mapped and attempted to identify the putative tumor suppressor gene in this region (3, 11, 12, 13, 14, 20, 21, 22). It is most likely that the putative tumor suppressor locus lies in a region on chromosome 19q13.3 between D19S241E (centromeric) and D19S596 (telomeric), spanning approximately 1.6 Mb. Additional evidence suggests an even smaller deletion area of 150 kb, containing the clustered genes *GTSCR1*, *EDH2*, *GTSCR2*, *SW*, *CRX*, and *STD* (17). The distance in a centromeric direction from this gene cluster to the closest microsatellite marker (D19S606) is approximately 300 kb, while the closest telomeric marker (D19S596) is 865 kb away. However, since *GTSCR1*, *EDH2*, *GTSCR2*, *SW*, *CRX*, and *STD*

have been excluded, and since most 19q deletions affect a larger telomeric region of the chromosome, the putative tumor suppressor gene candidate region has been expanded to include D19S596. One of the closest genes (115 kb telomeric to the gene cluster) is *PLA2G4C*.

While studying the *PLA2G4C* gene, we identified 3 allelic variants, *PLA2G4C*^{S}, *PLA2G4C*^{M} and *PLA2G4C*^{L}, which contain 1, 2, or 3 copies of a 27-bp repeat, respectively. Because these 3 alleles are moderately frequent in the general population, we were able to use *PLA2G4C*^{E1P2} as a minisatellite marker. The locus is informative in 55.7% of the CEPH cases and 57.8% of all tested cases. Because the repeat is a minisatellite of 27 bp, rather than a microsatellite of a few bp, the different alleles can be detected using ethidium bromide-stained agarose gels. This obviates the need for radioactive labeling and sequencing gels, offering distinctly practical advantages for diagnostic testing.

The *PLA2G4C*^{E1P2} polymorphism could also be useful for research purposes. *PLA2G4C* is the closest gene to the putative small deletion area containing *GTSCR1*, *EDH2*, *GTSCR2*, *SW*, *CRX*, and *STD* (17). The distance of *PLA2G4C* from this region is approximately 115 kb, whereas the next informative telomeric marker (D19S596) is 865 kb distant. The *PLA2G4C*^{E1P2} polymorphism could facilitate detection of small 19q deletions affecting the more telomeric candidate region. Since mutations have not been found in genes within the small deletion area, the telomeric end of the larger candidate region has now attracted great interest. *PLA2G4C* falls within this region, rendering it useful for further mapping studies.

PLA2G4C, in addition to its well-described role in inflammatory cascades, may be involved in the p53-regulated apoptotic pathway (4). This latter putative role raises the possibility that *PLA2G4C* could be a glioma suppressor gene. Interestingly, the 27-bp variations in *PLA2G4C* exon 1 (*PLA2G4C*^{S}, *PLA2G4C*^{M}, and *PLA2G4C*^{L}) fall within the 5′- untranslated region of the gene. The only known homology to this imperfectly palindromic repeat is found in an initiator sequence of the human *h-erg* potassium channel cDNA (9). This initiator sequence contains 23 of the 27 bp, and is perfectly preserved in its mouse homolog, *Merg1a*. These sequence similarities suggest that the region containing the 27-bp repeat could regulate *PLA2G4C* transcription. Furthermore, since the beginning of the 27-bp repeat can be shifted up to 5 bp downstream and still retain the same repeating sequence, alterations caused by the additional repeats or by a shift in the start of the repeat sequence could affect regulatory sequences and reduce *PLA2G4C* expression. Future studies could address the possible role of this polymorphic stretch in gene regulation. Regardless of whether the polymorphic repeat affects expression, the identification of this marker provides a convenient assay for determination of allelic loss within the most likely candidate region on 19q13.3.

Acknowledgments

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