Biomarker Insights

METHODOLOGY

Detection of 1p19q Deletion by Real-Time Comparative Quantitative PCR

Abhishek Chaturbedi[1], Liping Yu[2], Mark E Linskey[1] and Yi-hong Zhou[1]

[1]Department of Neurological Surgery, University of California, Irvine, CA. [2]Ziren Research LLC, Irvine, CA.
Corresponding author email: yihongz@uci.edu

Abstract: 1p/19q (1p and/or 19q) deletions are prognostic factors in oligodendroglial tumors (OT) and predict better survival after both chemotherapy and radiotherapy. While studying 1p/19q status as a potential variable within multivariate prognosis models for OT, we have frequently encountered unknown 1p/19q status within our glioma sample database due to lack of paired blood samples for loss of heterozygosity (LOH) assay and/or failure to perform fluorescence in situ hybridization (FISH). We realized that a 1p and 19q deletion assay that could be reliably performed solely on tumor DNA samples would allow us to fill in these molecular biology data "holes". We built recombinant DNA with fragments of the selected "marker" genes in 1p ($E2F2$, $NOTCH2$), and 19q ($PLAUR$) and "reference" genes ($ERC2$, $SPOCK1$, and $SPAG16$) and used it as quantification standard in real-time PCR to gain absolute ratios of marker/reference gene copy numbers in tumor DNA samples, thus called comparative quantitative PCR (CQ-PCR). Using CQ-PCR, we identified 1p and/or 19q deletions in majority of pure low-grade oligodenroglioma (OG) tumors (17/21, 81%), a large portion of anaplastic oligodendroglioma (AO) tumors (6/15, 47%), but rarely found in mixed oligoastrcytomas (OA) tumors (1/8, 13%). These data are consistent with results of LOH and FISH assays generally reported for these tumor types. In addition, 15 out 18 samples showed concordant results between FISH and CQ-PCR. We conclude that CQ-PCR is a potential means to gain 1p/19q deletion information, which prognostic and predictive values of CQ-PCR-derived 1p/19q status will be determined in a future study.

Keywords: glioma, oligodendroglial tumors, glioblastoma multiforme, 1p19q deletion, real-time comparative quantitative PCR

Introduction

The current gold standard for the diagnosis of glioma relies on histological examination of tumor tissue; however molecular analyses have uncovered important genetic abnormalities, some of which provide useful information pertaining to variations in patient's overall survival and/or response to treatment. Among gliomas, deletion of 1p and 19q are associated with tumors with oligodendroglial components.[1,2] 1p and/or 19q (1p/19q) deletions have been observed in up to 70% of oligodendrogliomas and 50% of mixed oligoastrocytomas (OA).[3,4] In addition to their diagnostic relevance, 1p/19q status is a predictive marker for overall improved outlook for patients with oligodendroglial tumors (OT).[3–6] Several studies have suggested that the 1p/19q status (co-deleted or not) predicts response of OT to radiation,[7] chemotherapy,[8–10] or combined.[11,12]

Due to the potential importance of 1p/19q status for patients with OT, several molecular methods have been standardized to detect this genetic abnormality in both clinical and basic research settings. The most commonly applied techniques to detect 1p/19q deletion are loss of heterozygosity (LOH),[13,14] and fluorescence in situ hybridization (FISH).[15,16] LOH requires paired blood DNA from the same patient, which is not routinely collected by most university tissue banks. FISH detection of 1p/19q deletion is frequently employed in clinical laboratories, but is costly and requires highly experienced personnel to ensure accuracy and data standardization.

To date, the best means for incorporating 1p/19q data into clinical practice remains controversial. There is an urgent need for accurate prognostic tools (particularly if the prognostic variables predict therapeutic response), for life-planning purposes for patients and treatment planning purposes for clinicians. However, development of prognosis models for gliomas has been a challenge due to their relatively low incidence and greater variation in survival per patient within a given histology and grade.[17] A good statistical percept for building a multivariable prognosis model is that we should have at least 10 events (deaths) for each candidate variable studied.[18] Thus when possible all available tumor resource of OT should be utilized. However, as shown in a recent retrospective study of over 1000 WHO grade III OT, 38% of tumor samples were missing 1p or 19q data. As a result, these tumors were excluded from the study.[12]

To explore an accurate means of filling these 1p 19q "data holes" independent of LOH or FISH via a test solely reliant on tumor sample DNA, we explored real-time PCR technology. We employed a standard-based absolute real-time PCR concept in quantifying gene expression,[19] to gain absolute ratios of DNA copy numbers of marker genes in 1p or 19q and reference genes in relatively "stable" areas in chromosomes, here referred as comparative quantitative PCR (CQ-PCR). Over the past decade, the regions of interest on 1p deletion have been mapped to 1p36.3, 1p36.1-p36.2, and 1p34-p35, the region of interest in 19q is located between 19q13.11 and 19q13.4,[20,21] and current clinical FISH test probes are associated with 1p36 and 19q13 regions.[5,16,22] *CAMTA1* and *NOTCH2* genes reside in 1p36.31-p36.23 and 1p13–11, respectively, with potential tumor suppression functions,[23,24] while *E2F2* tumor suppressor gene resides in 1p36.

Thus, we selected these three genes to analyze for deletion of 1p in gliomas. We selected *PLAUR* residing in 19q13 to study DNA copy number variation (CNV) of 19q, for its well-studied function in glioma invasion. According to CGH, there are certain chromosomes that typically remain unaltered during the course of glioma tumorigenesis, including 2q, 3p, 5q, 8q, 12q and 21p.[25–27] Hence three genes (*ERC2*, *SPAG16* and *SPOCK1*) located in chromosomes 3p14.3, 2q34 and 5q31, respectively, were selected as reference genes to determine CNV of the selected marker genes in 1p and 19q. We report a quantitative, sensitive, accurate, and relatively inexpensive assay for determining gene CNV in 1p/19q by real-time CQ-PCR that can be performed using only a few ng tumor DNA sample, independent of the need for LOH or FISH analysis.

Materials and Methods
Glioma and paired blood DNA samples

Glioma specimens were obtained from patients in accordance with Institutional Review Board approval at each participating institutions (University of California, Irvine, USA and University of Arkansas for Medical Sciences, USA). We included DNA samples from 44 OTs, 9 paired blood lymphocytes collected from patients during surgical resection of their gliomas, and 14 glioblastoma multiformes (GBMs) in this study. Breaking down of 44 OT samples included in analysis are 21 WHO grade II

oligodendroglioma (OG), 8 WHO Grade II mixed oligo-astrocytoma (OA), 15 either WHO grade III anaplastic oligodendroglioma (AO) or WHO grade III anaplastic mixed oligo-astrocytoma (AOA). For each case, histological assessment of the tumor tissues were performed by the neuropathologists in the participating institutes. All DNA samples were extracted from frozen glioma specimens using DNeasy kit (QIAGEN), and approximately 2 ng DNA in 10 mM Tris-HCl (pH 7.5) was used to run real time PCR for each gene.

Real-time CQ-PCR

CQ-PCR standard CQ-103 and 10X PCR primer mix for selected marker genes in 1p (*CAMTA*1, *E*2*F*2 and *NOTCH*2), 19q (*PLAUR*) and reference genes (*ERC2*, *SPAG16* and *SPOCK1*); and CQ-PCR standard CQ-108 and 10X PCR primer mix for *NLGN*4*Y* and the same set of reference genes are from Ziren Research (Irvine, CA). We run real-time PCR with Roche Light-Cycler real-time PCR instrument 2.0 (Indianapolis, IN) in a capillary tubes with 4 µl of standard or sample DNA (0.5 ng/µl), 1 µl of 10X FAST-START DNA Master SYBR Green I enzyme mix (Roche, Indianapolis, IN), 1 µl of the 10 X primer mix, and 4 µl of water in standard program setting with denature temperature set at 62°C, extension time 12 sec. A melting curve cycle was included for each run to endure no formation of primer dimer after 45 cycles. Gene copy numbers were exported directly from real-time PCR program, which was based on the standard curve (R value >0.99), a regression curve of the "cycle" numbers of four diluted standards when PCR reaching log amplification to their DNA quantifies (10^2, 10^3, 10^4, 10^5 copies). All genes were quantified at least twice in separate runs, to show assay reproduction with <20% standard deviation (SD), then mean copy number of each gene was taken for analysis, by calculating the ratio of each marker to the reference gene.

FISH detection of 1p and 19q loss

Paraffin embedded tissue obtained following surgical resection of glioma from patients were sent to Mayo Clinic, Department of Lab Medicine and Pathology, Rochester, Minnesota, USA for performing FISH with test probes (1p36 and 19q13) and control probes (1q24 and 19p13). FISH signals for each gene-specific FISH

probes were assessed under fluorescence microscope and the ratio of the probes for 1p/1q and 19p/19q were enumerated in 100 non-overlapping nuclei. A normal ratio is considered 1.0 and any ratio <0.80 is considered deletion of the region of interest.

Results

The principle of CQ-PCR as a modification of single-copy-gene standard-based real time PCR is via using a multi-gene-containing recombinant DNA standard that allows determination of absolute ratios of marker to reference gene copy numbers in sample DNA. A ratio of 1:1 between selected marker and reference genes in autosomal chromosomes is expected in normal cells while changes in this ratio in tumor DNA would suggest CNV, either deletion or amplification, in the studied gene of interest.

Considering the inherent genome instability of cancer cells, we analyzed the stability of three reference genes in tumor samples and found amplification of *SPAG16* in some OT. To mitigate this, we took the average of two ratios of *ERC2* and *SPOCK1* for most tumors. For other samples, the two reference gene ratios showing the most concordance were used to take a mean and SD.

With consideration of 10%–20% variation inherited with real-time PCR, the mean values of the marker and reference ratio was taken for determination of deletion (<0.8) or amplification (>1.2), Shown in Table 1, there was a gain at the 1p marker gene *CAMTA1* (1p36.31-23) in both GBM and OT, which were not found in other two 1p marker genes *E2F2* (1p36) and *NOTCH2* (1p13-p11). Thus average of these two 1p marker genes ratio to reference gene were taken to determine 1p deletion status (value <0.80 is considered 1p deleted). Based on the results from 67 samples, the mean (medium) of the SDs from different reference ratios for the four marker genes is 10%–15% (7%–9%), which supports our setting of cut-off-line of 0.8 or 1.2 in determining gene CNV from results of CQ-PCR.

Similarly, the ratio of DNA copy number between a gene in Y-chromosome and a gene in autosomal chromosome is expected to be 0.5 in a normal male diploid genome while zero in female DNA. As shown in Figure 1. CQ-PCR determination of absolute ratio of two gene copy numbers have given rise to a ratio value of 0.4–0.5 in normal cells of male

Table 1. CQ-PCR-derived Mean (SD) ratios of 1p/19 marker genes to each reference genes.

WHO	Histology	Tumor	1p36.31–23 *CAMTA1*	1p36 *E2F2*	1p13-p11 *NOTCH2,*	*Ave E2F2 & NOTCH*	19q13 *PLAUR*	FISH 1p	19q
IV	GBM	UCI-G22	**2.0 (0.54)**	**0.5 (0.27)**	1.1 (0.66)	**0.8**	**0.6 (0.38)**	na	na
IV	GBM	UCI-G24	1.0 (0.06)	0.8 (0.01)	1.0 (0.03)	**0.9**	**0.5 (0.01)**	na	na
IV	GBM	UCI-G25	0.9 (0.17)	1.0 (0.14)	1.2 (0.19)	**1.1**	**0.7 (0.09)**	na	na
IV	GBM	UCI-G26	**2.6 (1.30)**	0.7 (0.44)	1.0 (0.61)	**0.9**	**0.6 (0.33)**	na	na
IV	GBM	UCI-G27	1.3 (0.09)	1.0 (0.04)	1.1 (0.11)	**1.1**	0.8 (0.08)	na	na
IV	GBM	UCI-G28	**1.9 (0.21)**	1.3 (0.10)	1.2 (0.12)	**1.2**	0.9 (0.07)	na	na
IV	GBM	UCI-G29	1.2 (0.02)	0.9 (0.01)	1.1 (0.01)	**1.0**	**0.6 (0.01)**	na	na
IV	GBM	UCI-G30	0.9 (0.01)	1.0 (0.02)	1.0 (0.00)	**1.0**	**0.7 (0.01)**	na	na
IV	GBM	UCI-G31	0.9 (0.01)	0.9 (0.09)	1.2 (0.15)	**1.1**	1.0 (0.10)	na	na
IV	GBM	UCI-G32	1.2 (0.33)	1.2 (0.38)	1.4 (0.41)	**1.3**	0.8 (0.24)	na	na
IV	GBM	UCI-G33	**2.4 (0.51)**	**1.4 (0.01)**	**1.4 (0.01)**	**1.4**	1.0 (0.00)	na	na
IV	GBM	UCI-G34	**1.5 (0.13)**	0.9 (0.02)	0.9 (0.04)	**0.9**	**0.5 (0.03)**	na	na
IV	GBM	UCI-G35	**1.7 (0.21)**	**1.5 (0.16)**	0.9 (0.05)	**1.2**	1.3 (0.15)	na	na
IV	GBM	UCI-G36	1.0 (0.00)	0.9 (0.10)	1.2 (0.05)	**1.0**	**0.7 (0.08)**	na	na
III	AOA	UCI-O51	**1.6 (0.27)**	1.0 (0.22)	1.1 (0.18)	**1.1**	**0.6 (0.11)**	na	na
III	AO	UCI-O11	**2.9 (0.43)**	0.9 (0.13)	1.1 (0.16)	**1.0**	1.2 (0.21)	na	na
III	AO	UCI-O16	**1.8 (0.42)**	1.3 (0.30)	**0.2 (0.04)**	**0.7**	1.2 (0.30)	na	na
III	AO	UCI-O15	**0.5 (0.19)**	**0.2 (0.09)**	**0.3 (0.11)**	**0.3**	**0.4 (0.17)**	na	na
III	AO	UCI-O17	**1.7 (0.24)**	**0.7 (0.10)**	1.0 (0.15)	**0.8**	**0.5 (0.06)**	na	na
III	AO	UCI-O19	**3.3 (0.51)**	**1.6 (0.24)**	**0.2 (0.04)**	**0.9**	**0.7 (0.13)**	nml	del
III	AO	UCI-O20	1.2 (0.13)	1.2 (0.13)	0.9 (0.10)	**1.1**	**1.4 (0.11)**	na	na
III	AO	UCI-O27	**2.2 (0.17)**	**1.4 (0.10)**	1.2 (0.10)	**1.3**	1.2 (0.12)	nml	nml
III	AO	UCI-O32	0.9 (0.05)	1.0 (0.06)	1.0 (0.07)	**1.0**	0.8 (0.06)	nml	del
III	AO	UCI-O35	1.0 (0.09)	1.0 (0.09)	0.8 (0.07)	**0.9**	0.9 (0.09)	del	del
III	AO	UCI-O36	0.8 (0.05)	1.2 (0.08)	1.0 (0.07)	**1.1**	1.0 (0.08)	del	nml
III	AO	UCI-O42	1.0 (0.28)	1.0 (0.26)	**0.7 (0.19)**	**0.8**	**0.7 (0.21)**	del	del
III	AO	UCI-O52	1.2 (0.07)	0.9 (0.02)	1.1 (0.05)	**1.0**	0.8 (0.02)	nml	nml
III	AO	UAMS-O1	1.2 (0.13)	0.8 (0.09)	1.2 (0.13)	**1.0**	**0.6 (0.07)**	na	na
III	AO	UAMS-O7	**2.4 (0.04)**	1.0 (0.03)	1.0 (0.02)	**1.0**	1.2 (0.02)	na	na
II	OA	UCI-O26	1.0 (0.17)	1.0 (0.16)	1.1 (0.18)	**1.0**	0.8 (0.15)	nml	nml
II	OA	UCI-O31	1.2 (0.09)	**0.7 (0.06)**	0.9 (0.08)	**0.8**	0.9 (0.09)	na	na
II	OA	UCI O44	0.8 (0.02)	0.9 (0.01)	1.0 (0.01)	**0.9**	0.9 (0.00)	na	na
II	OA	UCI O46	0.9 (0.06)	0.9 (0.03)	1.1 (0.06)	**1.0**	0.9 (0.03)	na	na
II	OA	UCI O47	1.2 (0.13)	0.9 (0.07)	0.9 (0.09)	**0.9**	0.8 (0.06)	nml	nml
II	OA	UCI O50	0.8 (0.25)	**1.5 (0.06)**	**1.6 (0.10)**	**1.6**	**1.4 (0.06)**	na	na
II	OA	UAMS-O2	1.1 (0.07)	**0.7 (0.05)**	**0.7 (0.04)**	**0.7**	0.8 (0.05)	na	na
II	OA	UAMS-O3	**1.3 (0.14)**	1.1 (0.09)	1.1 (0.10)	**1.1**	0.9 (0.09)	na	na
II	OG	UCI-O10	0.8 (0.05)	**0.4 (0.02)**	**0.6 (0.03)**	**0.5**	**0.4 (0.03)**	na	na
II	OG	UCI-O12	**0.6 (0.09)**	**0.1 (0.01)**	**0.1 (0.01)**	**0.1**	**0.2 (0.03)**	na	na
II	OG	UCI-O13	0.8 (0.06)	**0.4 (0.03)**	**0.1 (0.01)**	**0.3**	**0.3 (0.03)**	na	na
II	OG	UCI-O14	1.2 (0.12)	**0.7 (0.08)**	0.8 (0.09)	**0.8**	**0.5 (0.05)**	nml	nml
II	OG	UCI-O18	**0.7 (0.11)**	**0.6 (0.10)**	**0.7 (0.11)**	**0.7**	1.0 (0.12)	nml	nml
II	OG	UCI-O21	**1.6 (0.05)**	0.9 (0.02)	**0.2 (0.01)**	**0.6**	**0.6 (0.02)**	na	na
II	OG	UCI-O24	1.1 (0.09)	**0.5 (0.05)**	**0.7 (0.06)**	**0.6**	**0.4 (0.04)**	del	del
II	OG	UCI-O28	**0.7 (0.16)**	1.1 (0.24)	1.1 (0.24)	**1.1**	**1.5 (0.29)**	nml	nml
II	OG	UCI-O29	**1.5 (0.10)**	**1.3 (0.09)**	1.2 (0.08)	**1.2**	**0.7 (0.07)**	nml	nml
II	OG	UCI-O34	0.7 (0.04)	0.8 (0.04)	0.9 (0.05)	**0.9**	1.2 (0.09)	nml	nml
II	OG	UCI-O38	1.1 (0.02)	1.1 (0.02)	0.9 (0.02)	**1.0**	1.2 (0.03)	nml	nml
II	OG	UCI-O37	1.1 (0.08)	0.8 (0.06)	**0.6 (0.05)**	**0.7**	**0.6 (0.06)**	na	na
II	OG	UCI-O39	**2.5 (0.18)**	**1.5 (0.11)**	**0.5 (0.04)**	**1.0**	1.1 (0.08)	na	na
II	OG	UCI-O41	0.9 (0.08)	**0.6 (0.06)**	**0.6 (0.060**	**0.6**	**0.5 (0.05)**	del	del
II	OG	UCI-O43	0.8 (0.01)	**0.5 (0.01)**	**0.7 (0.00)**	**0.6**	**0.5 (0.01)**	na	na
II	OG	UCI-O48	1.2.(0.29)	**0.7 (0.15)**	**0.9 (0.20)**	**0.8**	**0.6 (0.12)**	nml	nml

(*Continued*)

Table 1. (*Continued*)

WHO	Histology	Tumor	1p36.31–23 CAMTA1	1p36 E2F2	1p13-p11 NOTCH2,	Ave E2F2 & NOTCH	19q13 PLAUR	FISH 1p	19q
II	OG	UAMS-O4	1.6 (0.32)	1.4 (0.19)	1.4 (0.29)	1.4	0.7 (0.14)	na	na
II	OG	UAMS-O5	0.8 (0.06)	0.8 (0.04)	0.8 (0.07)	0.8	0.4 (0.04)	na	na
II	OG	UAMS-O6	0.7 (0.02)	0.6 (0.01)	0.6 (0.02)	0.6	0.3 (0.01)	na	na
II	OG	UAMS-O8	1.1 (0.04)	0.8 (0.03)	0.8 (0.03)	0.8	0.5 (0.02)	na	na
II	OG	UAMS-O9	1.6 (0.25)	1.1 (0.13)	1.1 (0.19)	1.1	0.6 (0.09)	na	na
		Concordance between CQ-PCR and FISH data						83%	78%

Notes: Numbers in bold and highlighted with light grey color indicates deletion of the specified marker gene with ratio to the reference gene <0.80; numbers highlighted with dark grey indicates amplification of the specified marker gene with ratio to the reference gene >1.20.
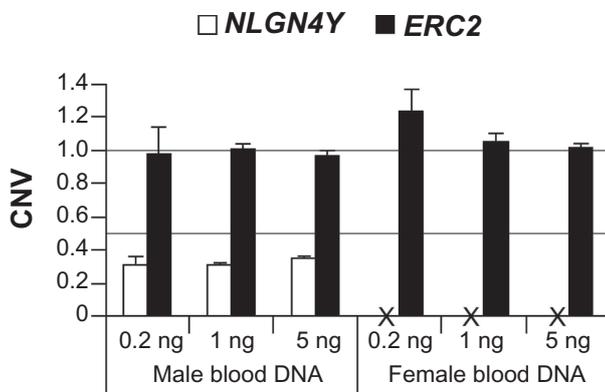**Abbreviations:** na, not available; wt, wild type; nml, normal; del, deletion.

and zero in female between genes in Y-(*NLGN*4*Y*) and autosomal chromosomes, while 1.0 between two autosomal genes (*ERC2*, *PGCP*, *SPAG16* and *SPOCK1*). To control human error in managing the tissue bank, we first carried out CQ-PCR of *NLGN*4*Y* and the autosomal genes in 61 tumor and 9 paired blood DNA samples, and analyzed for concordance with gender information provided by each participating institute's tissue bank. Out of total 70 DNA samples we identified 3 tumor samples with conflicting information between gender-associated gene in DNA and gender information of the patients, which were excluded from this study.

Blood sample is generally considered to be normal, in terms of mutations and allelic loss or amplification in the tumor sample. It is the reference used by loss of heterozygosity assay (LOH) for detecting loss of a microsatellite allele. In our CQ-PCR, we selected single copy protein-coding gene for

Figure 1. Real-time CQ-PCR detection of gender information based on human DNA samples. Bar and line height are mean (SD) ratios of DNA copy numbers of *ERC2* gene in 3p14.3 (*open bar*) and *NLGN4Y* gene in Yq11.221 (*filled bar*) to three other genes in 8q22.2 (*PGCP*), 2q34 (*SPAG16*), 5q31.2 (*SPOCK1*), in a male and a female blood DNA.

both marker and reference genes, detected the copy numbers of each, and compared relative difference in quantity. If there is deletion or amplification of a gene that is associated with tumor, it normally should not occur in normal lymphocyte. Our CQ-PCR detected deletions of 1p (*E2F2*, *NOTCH2*) and 19q (*PLAUR*) markers in 5/9 OT but none in the paired blood DNA samples; results for 6 tumor DNA samples with their paired blood DNA 1p and 19q were shown in Figure 2. *CAMTA1* was deleted in two and amplified in other two of the the six OT, but not in the paired blood DNA samples.

Based on average of *E2F2* and *NOTCH2* for 1p and *PLAUR* for 19q, as shown in Tables 1 and 2, our CQ-PCR revealed 1p/19q deletion for 57% OTs overall (25/44). Deletion of the 1p marker genes were not seen in the 14 GBM DNA samples, while deletion of the 19q marker gene was found in 8 out 14 (57%) GBM samples but not found in the eight OA samples. Thus, we did not find co-deletions of 1p and 19q in our GBM or OA samples. Deletion of either 1p, or 19q marker genes were mainly found in OG (48% and 76%, respectively). Co-deletions of 1p and 19q were also mainly found in OG (9/21, 43%), compared with AO & AOA combined (only 1 of 15, 7%).

The attempt of this study is to develop a PCR-based detection of 1p19q deletion for use in predicting patient's outcome. While FISH-based 1p19q deletion data have being shown with values in prognosis and prediction of radiation and chemotherapy, we therefore compared our CQ-PCR-derived data with that of FISH. Shown in Table 1, out of 18 DNA samples which had 1p19q deletion status available by both CQ-PCR and FISH techniques, the concordance rate between the two techniques was 83%
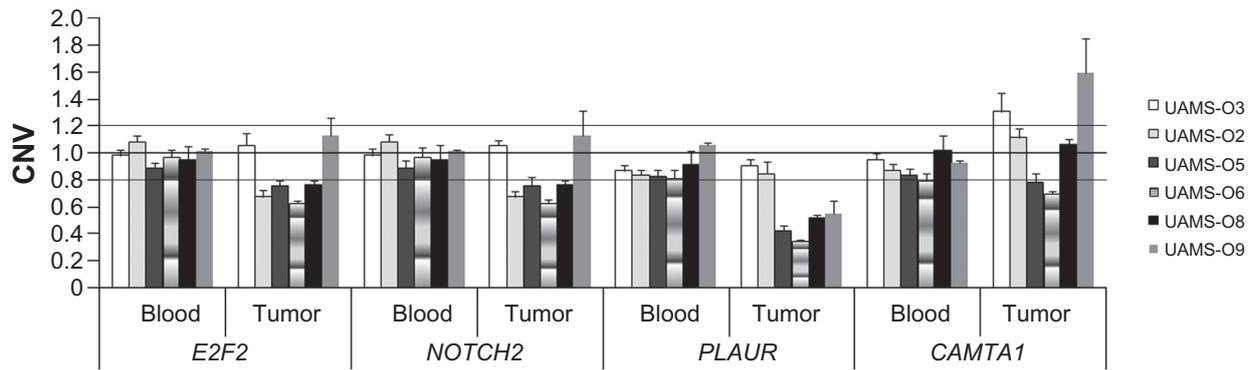
Figure 2. Comparison of gene copy number variation between paired DNA samples from patient's blood and tumor. Bar and line height are mean (SD) ratios of DNA copy numbers of *E2F2, NOTCH2, and PLAUR* to two reference genes (*ERC2* and *SPOCK1*), quantified by real-time CQ-PCR.

(15/18) and 78% (14/18) for 1p and 19q deletions, respectively.

Discussion

Our CQ-PCR detected 1p and/or 19q deletions in majority of classical oligodendroglioma samples (81% of OG and 47% of AO), but in minority of OA (13%). These results data consistent with those generally reported for LOH and FISH assays studying these tumor types.[3,4,23] In addition, 15 out 18 samples showed concordant results between FISH and CQ-PCR. Considering the small tumor sample size and limited marker loci used in our study, such a high concordance is remarkable, and suggests that our CQ-PCR has the potential to be used to define 1p/19q deletion status independent of FISH and LOH analysis. CQ-PCR does not require paired blood samples, strong reliance on technical considerations such as technician consistency and experience, and is much less costly than either LOH or FISH. Thus we could use this technology to effectively screen all available tumor DNA samples to determine 1p/19q deletion status which will aid in building multivariate prognosis models for OT. The prognostic and predictive values

of CQ-PCR-derived 1p/19q status will be determined based on a larger sample size with a large enough event number.

Our CQ-PCR finding of no 1p deletions in GBMs is consistent with results reported with LOH and FISH. However, we did detect *PLAUR* deletion in19q in 57% of the GBM samples, which has not been previously recognized. In contrast to GBM samples, none of the 8 OA samples had deletion of *PLAUR*. The identification of gain at 1p marker genes *CAMTA*1, *E2F*2 and *NOTCH*2 and 19 q marker gene *PLAUR* was not the focus of this study; however, in many OT and GBM samples, gains were detected by CQ-PCR that were not evident by FISH. Their biological implications and prognostic/predictive values on survival and treatment response are yet to be determined.

There have being prior explorations on obtaining 1p/19q status based solely on tumor DNA. High throuput genomic DNA microarray-based technology known as comparative genomic hybridization is able to detect 1p/19q deletions in gliomas, in addition to providing information on DNA CNV in the entire genome.[28,29] It requires well

Table 2. Frequency of 1p/19 deletions in gliomas.

Histology	Glioblatoma multiforme (GBM)	Anaplastic oligodendroglioma (AO), anaplastic oligo-astrocytoma (AOA)	Oligo-astrocytoma (OA)	Oligodendroglioma (OG)
WHO grade	IV	III	II	II
Tumor number	n = 14	n = 15	n = 8	n = 21
1p del	0%	14%	13%	48%
19q del	57%	33%	0	76%

trained personnel to not only perform the assay, but also process the data; thus incurring high cost in detecting a few known marker loci. Other PCR-based assays have also emerged including quantitative microsatellite analysis (QUMA) [30,31] and multiplex ligation-dependent probe amplification (MLPA).[32] QUMA uses PCR amplification of microsatellite loci that contain (CA)n repeats to determine CNV of genes of interest, and has also been validated with FISH technology on detection of 1p and 19q loss in oligodendroglioma.[31] However use of primers to amplify microsatellite DNA in quantitative PCR can be noisy, as multiple lengths of PCR product can be produced from cells even with homozygous loci, and amplification efficiency of different microsatellite markers can be different, thus the comparability between different platforms would be challenged. MLPA-based detection of 1p/19q has the advantage of detecting copy number changes of up to 45 loci in one relatively simple PCR based assay but it is semi quantitative and has yet to be widely adopted.[32]

Our real-time CQ-PCR utilizes multi-gene recombinant standard DNA to determine the absolute ratio of DNA copy numbers between the marker genes and multiple reference genes in tumor specimens. Both marker and reference genes were selected for their locations of interest and single-copy in genome with PCR primers designed to avoid amplification of corresponding pseudogenes in genome. It has advantages offered by QUMA in that all loci are informative, paired normal tissue from the same patient is not required, and even gain can be distinguished from loss. In comparison to QUMA, which requires accurate input of tumor DNA quantity, result from CQ-PCR is an absolute ratio of two gene's DNA quantity. Thus the result is independent of DNA input quantity and the accuracy is ensured by the specificity of PCR primers.

Our results showed that CQ-PCR is a highly sensitive test; 100 copy numbers of a specific gene were able to be robustly quantified in highly diluted DNA samples (0.5 ng/μL). In addition to determine loss (here 1p/19q), gain of gene copy numbers, such as *EGFR* was also be detected by CQ-PCR (our unpublished data).

Inherited with PCR-based molecular assay, this technique has the disadvantages that it will not detect events without causing an alteration of DNA copy number, such as chromosomal translations, and it relies on the stability of reference genes. The former concern is not an issue for detecting deletion of 1p/19q, while the later has been considered by selecting multiple reference genes residing in relatively stable areas of genome in specific cancer types, which information is based on whole genome comparative analyses. It still needs to be empirically validated based on a large number of tumor samples. In this study, we have tested four candidate reference genes in total 58 gliomas, with *ERC*2, *SPOCK*1, and/or *SPAG*16 giving similar results for the marker gene on CNV, while not when normalized with *PGCP*, a reference gene selected based on stable area of chromosome (data not shown). In this study, we have taken average ratios to *ERC*2, *SPOCK*1, and/or *SPAG*16 to determine deletion status of 1p/19q marker genes. The average (medium) of the SDs from different reference ratios for the four marker genes is 10%–15% (7%–9%), which verified our proper use of the reference genes.

In conclusion, CQ-PCR technology reported here could be used to effectively screen all available tumor DNA samples to get 1p/19q deletion status. Our prior studies of glioma prognosis have revealed a prognostic model for OT with high predictive accuracy that explained 63% survival variation with significant likelihood ratio *P*-value = 0.0076.[33] It is yet to be determined if the CQ-PCR-detected 1p/19q deletion has prognostic value, and furthermore if it improves the multivariable OT models we have established with combined clinical and gene expression variables.

Abbreviations
CQ-PCR, real time comparative quantitative polymerase chain reaction; CNV, copy number variations; FISH, fluorescence in situ hybridization; LOH, loss of heterozygosity.

Acknowledgment

Disclosures
Author(s) have provided signed confirmations to the publisher of their compliance with all applicable legal and ethical obligations in respect to declaration of conflicts of interest, funding, authorship

and contributorship, and compliance with ethical requirements in respect to treatment of human and animal test subjects. If this article contains identifiable human subject(s) author(s) were required to supply signed patient consent prior to publication. Author(s) have confirmed that the published article is unique and not under consideration nor published by any other publication and that they have consent to reproduce any copyrighted material. The peer reviewers declared no conflicts of interest.

References

1. Ransom DT, Ritland SR, Kimmel DW, et al. Cytogenetic and loss of heterozygosity studies in ependymomas, pilocytic astrocytomas, and oligodendrogliomas. *Genes Chromosomes Cancer*. Nov 1992;5(4):348–56.
2. Bello MJ, Vaquero J, de Campos JM, et al. Molecular analysis of chromosome 1 abnormalities in human gliomas reveals frequent loss of 1p in oligodendroglial tumors. *Int J Cancer*. Apr 15, 1994;57(2):172–5.
3. Felsberg J, Erkwoh A, Sabel MC, et al. Oligodendroglial tumors: refinement of candidate regions on chromosome arm 1p and correlation of 1p/19q status with survival. *Brain Pathol*. Apr 2004;14(2):121–30.
4. Okamoto Y, Di Patre PL, Burkhard C, et al. Population-based study on incidence, survival rates, and genetic alterations of low-grade diffuse astrocytomas and oligodendrogliomas. *Acta Neuropathol*. Jul 2004;108(1):49–56.
5. Smith JS, Perry A, Borell TJ, et al. Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas. *J Clin Oncol*. Feb 2000;18(3):636–45.
6. Jenkins RB, Blair H, Ballman KV, et al. A t(1;19)(q10;p10) mediates the combined deletions of 1p and 19q and predicts a better prognosis of patients with oligodendroglioma. *Cancer Res*. Oct 15, 2006;66(20):9852–61.
7. Bauman GS, Ino Y, Ueki K, et al. Allelic loss of chromosome 1p and radiotherapy plus chemotherapy in patients with oligodendrogliomas. *Int J Radiat Oncol Biol Phys*. Oct 1, 2000;48(3):825–30.
8. Cairncross JG, Ueki K, Zlatescu MC, et al. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. *J Natl Cancer Inst*. Oct 7, 1998;90(19):1473–9.
9. Hoang-Xuan K, Capelle L, Kujas M, et al. Temozolomide as initial treatment for adults with low-grade oligodendrogliomas or oligoastrocytomas and correlation with chromosome 1p deletions. *J Clin Oncol*. Aug 1, 2004;22(15):3133–8.
10. Levin N, Lavon I, Zelikovitsh B, et al. Progressive low-grade oligodendrogliomas: response to temozolomide and correlation between genetic profile and O6- methylguanine DNA methyltransferase protein expression. *Cancer*. Apr 15, 2006;106(8):1759–65.
11. Cairncross G, Berkey B, Shaw E, et al. Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: Intergroup Radiation Therapy Oncology Group Trial 9402. *J Clin Oncol*. Jun 20, 2006;24(18):2707–14.
12. Lassman AB, Iwamoto FM, Cloughesy TF, et al. International retrospective study of over 1000 adults with anaplastic oligodendroglial tumors. *Neuro Oncol*. Jun 2011;13(6):649–59.
13. Ino Y, Betensky RA, Zlatescu MC, et al. Molecular subtypes of anaplastic oligodendroglioma: implications for patient management at diagnosis. *Clin Cancer Res*. Apr 2001;7(4):839–45.
14. Reifenberger G, Louis DN. Oligodendroglioma: toward molecular definitions in diagnostic neuro-oncology. *J Neuropathol Exp Neurol*. Feb 2003;62(2):111–26.
15. Smith JS, Alderete B, Minn Y, et al. Localization of common deletion regions on 1p and 19q in human gliomas and their association with histological subtype. *Oncogene*. Jul 15, 1999;18(28):4144–52.
16. Reddy KS. Assessment of 1p/19q deletions by fluorescence in situ hybridization in gliomas. *Cancer Genet Cytogenet*. Jul 15, 2008;184(2):77–86.
17. CBTRUS. Statistical Report: Primary Brain and Central Nervous System Tumors Diagnosed in the United States in 2004–2006. *Central Brain Tumor Registry of the United States*. 2010.
18. Katz MH. *Multivariable Analysis*. Cambridge: Cambridge University Press; 1999.
19. Zhou YH, Raj VR, Siegel E, Yu L. Standardization of gene expression quantification by absolute real-time qRT-PCR system using a single standard for marker and reference genes. *Biomarker Insights*. 2010;5:79–85.
20. Jeuken JW, von Deimling A, Wesseling P. Molecular pathogenesis of oligodendroglial tumors. *J Neurooncol*. Nov 2004;70(2):161–81.
21. Hartmann C, Mueller W, von Deimling A. Pathology and molecular genetics of oligodendroglial tumors. *J Mol Med (Berl)*. Oct 2004;82(10):638–55.
22. van den Bent MJ, Carpentier AF, Brandes AA, et al. Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. *J Clin Oncol*. Jun 20, 2006;24(18):2715–22.
23. Barbashina V, Salazar P, Holland EC, Rosenblum MK, Ladanyi M. Allelic losses at 1p36 and 19q13 in gliomas: correlation with histologic classification, definition of a 150-kb minimal deleted region on 1p36, and evaluation of CAMTA1 as a candidate tumor suppressor gene. *Clin Cancer Res*. Feb 1, 2005;11(3):1119–28.
24. Boulay JL, Miserez AR, Zweifel C, et al. Loss of NOTCH2 positively predicts survival in subgroups of human glial brain tumors. *PLoS One*. 2007;2(6):e576.
25. Reifenberger J, Reifenberger G, Liu L, James CD, Wechsler W, Collins VP. Molecular genetic analysis of oligodendroglial tumors shows preferential allelic deletions on 19q and 1p. *Am J Pathol*. Nov 1994;145(5):1175–90.
26. Mohapatra G, Bollen AW, Kim DH, et al. Genetic analysis of glioblastoma multiforme provides evidence for subgroups within the grade. *Genes Chromosomes Cancer*. Mar 1998;21(3):195–206.
27. Ino Y, Silver JS, Blazejewski L, et al. Common regions of deletion on chromosome 22q12.3-q13.1 and 22q13.2 in human astrocytomas appear related to malignancy grade. *J Neuropathol Exp Neurol*. Aug 1999;58(8):881–5.
28. Jeuken JW, Sprenger SH, Wesseling P, et al. Identification of subgroups of high- grade oligodendroglial tumors by comparative genomic hybridization. *J Neuropathol Exp Neurol*. Jun 1999;58(6):606–12.
29. Jeuken JW, Sprenger SH, Boerman RH, et al. Subtyping of oligoastrocytic tumours by comparative genomic hybridization. *J Pathol*. May 2001;194(1):81–7.
30. Ginzinger DG, Godfrey TE, Nigro J, et al. Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. *Cancer Res*. Oct 1, 2000;60(19):5405–9.
31. Nigro JM, Takahashi MA, Ginzinger DG, et al. Detection of 1p and 19q loss in oligodendroglioma by quantitative microsatellite analysis, a real-time quantitative polymerase chain reaction assay. *Am J Pathol*. Apr 2001;158(4):1253–62.
32. Jeuken J, Cornelissen S, Boots-Sprenger S, Gijsen S, Wesseling P. Multiplex ligation-dependent probe amplification: a diagnostic tool for simultaneous identification of different genetic markers in glial tumors. *J Mol Diagn*. Sep 2006;8(4):433–43.
33. Zhou YH, Hess KR, Raj VR, et al. Establishment of prognostic models for astrocytic and oligodendroglial brain tumors with standardized quantification of marker gene expression and clinical variables. *Biomarker Insights*. 2010;5:153–168.