Technical Advance

Detection of 1p and 19q Loss in Oligodendroglioma by Quantitative Microsatellite Analysis, a Real-Time Quantitative Polymerase Chain Reaction Assay

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The combined loss of chromosomes 1p and 19q has recently emerged as a genetic predictor of chemosensitivity in anaplastic oligodendrogliomas. Here, we describe a strategy that uses a novel method of real-time quantitative polymerase chain reaction, quantitative microsatellite analysis (QuMA), for the molecular analysis of 1p and 19q loss in oligodendrogliomas and oligoastrocytomas in archival routinely processed paraffin material. QuMA is performed on the ABI 7700 and based on amplifications of microsatellite loci that contain (CA)_n repeats where the repeat itself is the target for hybridization by the fluorescently labeled probe. This single probe can therefore be used to determine copy number of microsatellite loci spread throughout the human genome. In genomic DNA prepared from paraffin-embedded brain tumor specimens, QuMA detected combined loss of 1p and 19q in 64% (21 of 32) of oligodendrogliomas and 67% (6 of 9) of oligoastrocytomas. We validate the use of QuMA as a reliable method to detect copy number by showing concordance between QuMA and fluorescence *in situ* hybridization at 37 of 45 chromosomal arms tested. These results indicate that QuMA is an accurate, high-throughput assay for the detection of copy number at multiple loci; as many as 31 loci of an individual tumor can be analyzed on a 96-well plate in a single 2-hour run. In addition, it has advantages over standard allelic imbalance/loss of heterozygosity assays in that all loci are potentially informative, paired normal tissue is not required, and gain can be distinguished from loss. QuMA may therefore be a powerful molecu-

lar tool to expedite the genotypic analysis of human gliomas in a clinical setting for diagnostic/prognostic purposes. (Am J Pathol 2001, 158:1253–1262)

Molecular analyses of human malignancies began with the observation that the distribution of chromosomes had been corrupted in the nuclei of cancer cells. Methods have since evolved to expose the smallest of genetic differences between normal and tumor cells, and the hope is that some of these lesions can be targeted therapeutically. Although specific genetic or expression patterns are the basis for the design of the next generation of cancer therapies, the chemosensitivity of tumors to existing regimens has already been associated with certain genetic alterations in some human cancers. An example of this was found in anaplastic oligodendroglioma, in which tumors showing combined loss of 1p and 19q exhibited a clinical behavior distinct from tumors in which 1p and 19q were intact.^{1,2} Specifically, patients whose tumors demonstrated 1p/19q loss lived significantly longer and their tumors were more sensitive to therapeutic agents. In addition, the clinical diagnosis of brain tumors is based primarily on morphological features, and a goal of molecular diagnosis would be to serve as an adjunct to histopathology with respect to classifying subtypes of brain tumors.

These are compelling results to use the molecular analysis of chromosomes 1p and 19q to assist in the histological diagnosis of human gliomas and clinical management of affected patients. Several molecular methods are currently used to detect 1p/19q loss in oligodendrogliomas in both clinical and basic research settings. Allelic imbalance, sometimes referred to as loss

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of heterozygosity (LOH),³ detects reduction from heterozygosity to homozygosity at 1p and 19q loci.^{1,4-6} The routine use of LOH to detect alterations at specific loci is limited by the requirements of paired normal tissue from the same patient and the presence of heterozygosity in the normal tissue (an informative locus). Fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization are established methods to detect copy number, but both are labor-intensive techniques.⁶⁻¹¹ Real-time quantitative polymerase chain reaction (PCR) is currently also used to determine copy number at genomic loci but, to date, only single-copy sequence-specific probes have been used.¹²⁻¹⁶ A novel modification of this technique that utilizes a probe designed to bind to CA repeats, quantitative microsatellite analysis (QuMA), has recently been described.^{17,18} QuMA is a high-throughput real-time PCR assay that may be adaptable as a molecular diagnostic technique, as it can be performed on DNA derived from either frozen or paraffin-embedded tissue in as little as 2 hours. In the initial report,¹⁷ the template DNAs were in general prepared from fresh or frozen tissue samples. On a practical level, many tumor samples are available only as formalin-fixed, paraffin-embedded tissues from pathology archives. Accordingly in this study, we determined whether DNA extracted from paraffin-embedded tissues would be a substrate of sufficient quality for QuMA. We then applied QuMA to 41 cases of oligodendroglioma/oligoastrocytoma, and assessed its reliability by comparing the results in a subset of cases with a well-established molecular technique, FISH.

Materials and Methods

DNA Preparations

Oligodendrogliomas and oligoastrocytomas were obtained as routinely processed paraffin-embedded samples from clinical material at the University of California, San Francisco. For each case, histological assessment of the tumor tissue to be used was performed by a neuropathologist (KA). If at least 90% of the area of the tissue was interpreted as tumor, the sections were directly cut from the block into an Eppendorf tube for DNA isolation. If the proportion of tumor was <90%, 10 to 20 sections were cut on glass slides, and the tumor tissue was hand dissected from the normal tissue. Tumor DNA was prepared from paraffin-embedded tissues by digesting deparaffinized tumor sections for 3 to 5 days with proteinase K at 55°C (0.5 mg/ml in 100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 25 mmol/L ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate), followed by a phenol:chloroform:isoamyl extraction and ethanol precipitation. Concentrations were determined on the ABI 7700 so that each DNA, when amplified with the reference locus, reached a threshold cycle equivalent to a 5-ng control DNA.

A.

B.

Figure 1. TaqMan chemistry and strategy for QuMA. **A:** Fluorescence is detected when the 5'-label of the probe, or reporter (R), is liberated from the 3'-quenching signal (Q) through the exonucleolytic activity of *Taq* polymerase. The probe for QuMA hybridizes to the CA repeat of microsatellite sequences. **B:** Schematic illustration of QuMA experiment comparing tumor DNA with loss (short-dashed lines) to normal DNA (long-dashed lines). The curves for the reference locus for two DNAs are superimposed and for simplicity are shown as a single curve (solid line). Comparison of each microsatellite curve with its appropriate reference is represented as the δCt , where $\delta Ct = Ct(\text{microsatellite}) - Ct(\text{reference})$, and Ct is cycle number at threshold. The relative copy number (tumor to normal DNA) is determined from the $\delta\delta Ct$, where $\delta\delta Ct = \delta Ct(\text{tumor}) - \delta Ct(\text{normal})$. The copy number in the tumor DNA is equal to $2 \times 2^{-\delta\delta Ct}$.

QuMA

All primer sets were used to perform amplifications in triplicate on the ABI 7700 instrument (Applied Biosystems, Foster City, CA). The probe for the detection of amplified product was a 21-bp oligomer complementary to the microsatellite CA repeat [5'-6-carboxy fluorescein (FAM)-TGTGTGTGTGTGTGTGTGTGT 3'-6-carboxy tetramethyl rhodamine (Integrated DNA Technologies, Coralville, IA)], rendering it capable of hybridizing to microsatellite loci spread throughout the human genome. The flanking primers, thus, could be continuously changed while the probe remained constant (Figure 1A). As FAM is released from the 5' end of the probe by *Taq* polymerase, fluorescence is detected and plotted as a function of PCR cycle number.^{12,19} Copy number is determined from the PCR cycle number (Ct) at which DNAs reach a threshold amount of fluorescence above background.

Reactions (50 μ l) were performed in 1 $\times$ PCR buffer A (Perkin Elmer, Foster City, CA), 2.5 mmol/L $MgCl_2$, 0.4 μ mol/L each primer, 200 μ mol/L each dNTP, 60 nmol/L probe, and 1.25 U AmpliTaq Gold. Cycling parameters were as follows: 95°C for 12 minutes $\times$ 1 cycle, (95°C for 20 seconds, 55°C for 20 seconds, 72°C for 45 seconds) $\times$ 40 cycles. A master mix of the components including the equivalent of 5 ng of genomic DNA per well (10 μ l at 0.5 ng/ μ l) was made and aliquoted into a 96-well

Table 1. Primer Sets Used for QuMA

Primer	Forward	Reverse
D1S468	TAAATATTAGGTCAAACCATG	ATGGCTGCATATAATGTTG
D1S214	CCGAATGACAAGGTGAGACT	AATGTTGTTTCCAAAGTGCC
D1S2736	TACCTCCAGGTATTCTTGG	TTTTGAGGTGTGAGAGCAG
D1S2783	CCCTACCCTAATCCACTG	GTTTATGTTTCACCTCTATCC
D1S514	TGAATGCGTGGTCCCAACAT	GACTCAGACTTCCATCTGGACT
D1S228	AACTGCAACATTGAAATGGC	GGGACCATAGTTCTTGGTGA
D1S199	GGTGACAGAGTGAGACCCTG	CAAAGACCATGTGCTCCGTA
D1S2620	AAGAGTTGTCCAACCAAATTG	GAATCAGGGATGGATGTG
D1S2892	CCCTGGGCAACATGGCAAG	CTGGGACCACAGAGCCACC
D1S224	CATAATCCTTTGGCCCAATC	CATCACACATTTTAGAATACAGTGC
D10S536	GGTCAAATTATCATTGTTTGTTC	AAGTTTTATCTCAAGACTGTTGCC
D10S1683	TTAAGTGCCAATGCCCAATC	TGCTAACACGACAGTATCCCAGAC
D19S424	AGCTGGTTATCTTGAGGGAG	TAGGCCACATGGAGGAGT
D19S408	AGCTCTATGGGGTGGTGCC	GCCTCTTAGAGTTTTGGGAG
D19S596	CCACAGAGCAAGACTCGAT	GCCAGAGCCACTGTGT
D19S867	CAATGAAAATGCTTTGTAAAAC	CCTTCAGAGGTGACCAG
D19S418	ACCAGGCATCCAGTGTTC	CAACTATCCCGCCTTTGT
D19S926	TCTGGTGAGAATTCCTAAGTAGTTC	GGCCTTATGCGTGAGTAGTT
Reference pool primers		
D2S385	AGCTGTCAGTAGAAATAAGCAGAGA	TCAATAACACGCCAAAAGAC
D3S1554	ATTCATCTTGTTACTGTTCAATTGT	GGGCAAACCCAAAGACT
D5S643	TGGGCGACAGAGCCATC	TGTGGTGTGCCATTTATTGACT
D8S1800	CCATCAAATGTCGAACACTG	GTCCACCAATGCGTTAAAG
D12S1699	ACCTCATGCCTGTTAGG	TTCGTTACATCCTGG
D21S1904	ATGAGTTCAGTGTTTCATGGACATC	AGCAAGATTACTGTCTGGTTCCC

optical plate. Ten μ l of the primer sets were subsequently added to designated wells.

To normalize for differences in the amount of total input DNA, amplification at a reference locus was performed once per plate in triplicate for each individual DNA. The maximum number of additional test loci that could therefore be analyzed on a single 96-well plate for one DNA is 31. To suppress the effect of possible deviations from the diploid state at a single locus in unstable tumor DNAs, the reference chosen was a multiplex PCR or amplification of a pool of microsatellite primer pairs in a single well, rather than any individual locus. For these experiments, six primer sets for the reference pool were chosen from chromosomes that typically remain unaltered during the course of glioma tumorigenesis:²⁰⁻²² 2q (D2S385), 3p (D3S1554), 5q (D5S643), 8q (D8S1800), 12q (D12S1699), and 21p (D21S1904) (Table 1). The final concentration of the pooled primers in the reference PCR wells is 0.4 μ mol/L for each of the 12 primers and reached Ct at one to four cycles before that of the test loci used in this study.

Choice of individual loci on chromosomes 1p, 10q, 19p, and 19q (Table 1) was dependent on the mean δ Ct (values between 1 to 4) and the SD (<0.25) (Figure 1B) derived from amplification of normal DNA for 8 to 10 unrelated individuals. Genomic DNAs to calculate the average δ Ct were isolated from paraffin-embedded normal brain tissue. To minimize potential amplification variability of a locus between different individuals, loci were selected that: 1) are ≤ 150 bp in length, 2) contain the full 10.5 CA repeats of the probe, and 3) have an efficiency of amplification that is $>90\%$ (Figure 2, A and B). PCR efficiency (E) can be determined from the slope of a four-point curve where the log of the input DNA mass is plotted against the threshold cycle (Ct). The equation of

this line is derived from $X_{Ct} = X_0(1 + E)^{Ct}$, where X_{Ct} = DNA quantity at the threshold (constant) and X_0 = quantity of input DNA. The slope of this line, S, is equal to $-1/\log(1 + E)$ or $E = 10^{(-1/S)} - 1$. Amplification of 5 ng for these experiments falls within the linear range of the plot of Ct against $\log X_0$ and reaches threshold at cycle numbers <32 .

Calculations

The Ct values for each set of triplicates were averaged. The Ct of the pooled reference was subtracted from the Ct for each locus to obtain the δ Ct [δ Ct = Ct (microsatellite) – Ct (reference)]. δ Ct values were determined for loci in tumor samples and a set of 10 normal genomic DNAs. The average of the 10 δ Ct values [δ Ct (normal)] measured from the normal DNAs was calculated once for each locus in this study and used in the subsequent calculations for all experiments performed on a single ABI 7700. The relative copy number of a locus in tumor DNA to normal DNA = $2^{-\delta\delta Ct}$, where $\delta\delta Ct$ = δ Ct (tumor) – δ Ct (normal). Because the copy number is 2 in normal diploid DNA, the relative copy number multiplied by 2 yields copy number in the unknown sample at the locus amplified (Figure 1B).

To determine whether the copy number calculated differs significantly from normal DNA so as to be called a loss or a gain, the pooled SD for all loci in normal DNAs was used to create a tolerance interval (TI) with a confidence of 95%.¹⁷ $TI = 2 \times 2$ to the power $\pm [2.28 \times \text{the square root of } \{\sum_i (n_i - 1) \times SD_i^2 / \sum_i (n_i - 1)\}]$ where n_i = number of normals analyzed per microsatellite locus and 2.28 is a two-sided tolerance limit factor for a total of 167

A.

B.

Figure 2. PCR efficiency of paraffin-embedded DNA. **A:** DNA from normal paraffin-embedded tissue was serially diluted starting at 50 ng, and a microsatellite locus from chromosome 1 was amplified. The diluted DNAs reach threshold as predicted from the fundamental equation for PCR, $X_n = X_0(1+E)^n$, or $X_n = X_02^n$ when efficiency (E) is 100%. For example, the cycle difference at threshold for the 20 ng and 5 ng quantities is 2 reflecting the fourfold difference in starting DNA amounts. **B:** Plot of the log of input DNA quantities versus C_t . The equation for this line is derived from $X_n = X_0(1+E)^n$ when $n = C_t$, cycle at threshold fluorescence. The efficiency of PCR can be calculated from the slope of the line, $E = 10^{-1/\text{slope}} - 1$. Because the PCR efficiency is near 100% for all of our primer sets, the fundamental equation for PCR [$X_n = X_0(1+E)^n$] can be assumed to be $X_n = X_02^n$. In the experiment shown, the data points have a correlation of 0.9996 with $E = 96\%$ demonstrating that amplification of paraffin-embedded DNA over this range of input DNA closely adheres to the fundamental equation for PCR.

measurements [$\sum_i (n_i - 1)$].²³ Based on this TI, copy numbers <1.58 were considered to be losses whereas those >2.53 were considered to be gains.

FISH

FISH was performed as previously described.⁶ Briefly, 5- μ m sections from paraffin-embedded tumors were deparaffinized, dehydrated in an ethanol series, microwave on high power for 10 minutes in 10 mmol/L citrate buffer, pH 6.08, and digested in pepsin (4 mg/ml in 0.9% NaCl, pH 1.5) at 37°C for 15 minutes. Bacterial artificial chromosomes (BACs) from 1p36, 1q24, 19p13, and 19q13.3 were directly labeled with fluorescent nucleo-

tides, and just before hybridization, probes and target DNA were simultaneously denatured at 80°C for 5 minutes. Hybridizations were performed at 37°C overnight, and subsequent washes were performed at 45°C as follows: 1.5 mol/L urea/0.1 $\times$ standard saline citrate, twice for 15 minutes; 2 $\times$ standard saline citrate, 5 minutes; 2 $\times$ standard saline citrate/0.1% Nonidet P-40, 5 minutes. After hybridization, the mean number of signals scored for each of the four probes was determined after counting 300 to 500 nuclei. A ratio between p and q arms was calculated for both chromosome 1 and chromosome 19. In general, for this set of tumors, the ratios (0.5 to 1.0) simply reflected loss or retention. A ratio of 0.85 or less was scored as a loss. A gain was scored on either chromosome when $>15\%$ of nuclei exhibited more than two signals for either a single probe or both.

Results

QuMA at 1p

To test whether PCR would be quantitative in paraffin DNA samples, serial dilutions were prepared from isolated normal and tumor DNAs from paraffin sections and subjected to real-time quantitative PCR at a single microsatellite locus (Figure 2A). Over a range of 1 ng to 50 ng, the starting DNAs reached threshold as predicted from the equation $X_n = X_02^n$ where $n = C_t$ and efficiency is 100% (Figure 2B). For example, the 20 ng and 5 ng quantities differed by exactly two cycles at the threshold, reflecting the fourfold difference in DNA mass.

Our analysis encompassed the pattern of loss at microsatellites along the entire 160 cM of 1p. Copy numbers at five 1p loci were determined for 41 tumors and are presented in Figure 3. In these experiments, 1p loss (as defined by copy number <1.58 in two or more sequential loci) was detected in 28 of 41 (68%) cases where an oligodendroglial component was histologically apparent. The entire 1p arm was lost in 23 cases. Loss limited to the two distal markers (D1S468 and D1S214) occurred in two cases (10896 and 7876) and interstitial deletions that encompassed the region previously identified were detected in two cases (8503 and 307). For 8503, the marker D1S228 at 32.4 cM was also retained. Deletion of consecutive markers in a nonoverlapping region of 1p (D1S2783 and D1S514) with retention of distal markers was found only in a single tumor (283). In addition to the losses detected, two cases (185 and 262) appeared to have extensive regions of gain along chromosome 1.

QuMA at 19p, 19q, and 10q

To gain insight into the alterations that appear in conjunction with chromosome 1p loss in our patient sample, QuMA was performed at loci on chromosomes 10q, 19q, and 19p. Deletion of chromosome 1p is prevalent in oligodendrogliomas that have sustained 19q loss,^{6,10,20} and chromosomal abnormalities of 10q have been associated with the development of higher grade gliomas, including glioblastomas.^{20,24,25} A single site on 19p, not

		1p					10q		19p	19q				
		1p36								19q13.3				
		DIS468	DIS214	DIS2736	DIS2783	DIS514	DIS536	DIS1683	DIS424	DIS408	DIS596	DIS867	DIS418	DIS926
		6.2	16.9	26.9	65.6	157.4	123.3	143.9	15.4	67.8	77.6	81.2	97.5	100.1
2286	AOA	0.96	0.86	1.13	0.97	1.19	1.77	2.09	2.33	0.92		1.15		
12463	AOA	0.64	0.91	1.00	0.85	0.85	1.91	1.64	1.41	0.93		1.01		
2819	AOA	0.8	0.89	1.16	0.83	1.19	1.51	2.04	2.22	1.05		1.15		
1222	O	1.19	0.99	0.99	0.88	0.90	2.24	1.94	1.77	0.88		1.13		
1207	AO	0.86	1.04	0.96	0.91	0.70	1.86	1.98	1.84	0.75		1.27		
1309	O	1.15	1.34	0.83	0.98	0.91	1.96	1.96	2.00	0.83		1.24		
1239	O	1.22	0.95	1.03	0.95	0.77	2.17	2.02	1.78	0.87		1.38		
5457	O	1.23	1.28	1.10	0.92	1.27	2.02	1.82	2.45	1.27	1.26	0.90	1.11	1.05
212	AOA	1.1	1.37	1.00	1.46	1.12	1.93	2.15	2.07	0.88		1.25		
8758	O	1.11	1.52	1.31	1.03	1.45	2.08	2.16	2.36	1.11		1.44		
3191	AO	0.92	0.88	0.86	0.91		1.67	1.62	1.67	0.79		1.20		
4507	O	1.26	1.29	1.05	1.06		1.87	1.97	2.00	1.03		1.24		
10612	O	1.27	1.31	0.94	0.84		2.11	1.79	2.24	0.75		1.13		
6829	O	1.44	1.48	1.49	1.18				1.84	1.18	1.15	2.22	2.52	2.12
8282	AO	0.85	1.09	1.07	1.27		2.43	1.46	1.29	0.85		1.01		
18253	AOA	1.17	1.55	1.06	0.97				2.07	0.71	0.96	1.53	1.30	1.17
2470	AO		0.86	0.79	0.85	1.29		1.77	2.29	1.06		0.78		
4541	AO	1.17	2.08	0.92	1.24	1.08	2.23	1.77	1.91	0.95		0.86		
11654	O	1.22	1.93	1.07	1.46	1.16	2.15	1.81	1.66	1.05		1.08		
105	AO	0.88		1.02	0.90		1.85	1.70	1.76	0.82		1.02		
1005	AOA	1.12		1.28	1.44		2.39	2.41		1.10	1.02	1.56	1.40	0.77
12730	AO	1.06		1.09	0.76		1.66	1.71	2.17	1.00	1.01	1.04	0.93	0.75
6/SC	AO	0.81		0.90	1.54		2.29	1.96	1.64	0.75		1.20		
10896	O	0.97	1.39	1.69	2.87	1.86	2.00	1.16		0.89		0.85		
7876	AO	1.18	1.11	1.90	1.92				2.17	1.16		1.36		
8503	AO	2.31	0.85	1.21	1.65	1.88	1.09	1.04	3.00	2.72		3.07		
307	AO	1.66	1.38	1.43	1.14	1.56	1.96	1.74	2.35	1.40	1.57	1.53	1.90	1.99
14670	O	1.35	2.26	2.32	1.90	1.40	2.46	2.36	3.37	2.06	1.60	3.54	3.05	3.07
283	AO	1.61	1.64	2.12	1.46	1.04	2.78	2.78	1.57	1.25	1.13	1.77	2.55	2.15
7312	OA	1.56		1.84	1.81		2.79	2.93		2.01		3.10		
227	O	1.90	2.82	1.53	2.19	2.17	1.54	1.49	2.82	1.71		2.13		
191	AO	2.03		2.13	2.61		2.22	1.92		1.00		1.06		
195	AO	1.65	2.20	1.86	3.93	2.07	2.72	1.77	1.88	0.84		1.29		
48	AOA	2.47	2.36	2.46	2.54	2.28	2.54	2.33	1.76	1.92		2.55		
1258	AO	2.13	1.80	1.75	1.80		1.67	1.19	2.21	1.69		2.32		
236	AO	2.73		1.91	1.77		1.47	1.18	1.44	1.68		2.08		
16223	O	2.08	2.36	2.53	2.78	2.44	2.63	2.47	1.99	1.95	1.55	3.09	2.50	2.08
185	O	3.12		3.10	3.30		3.13	2.53	2.54	3.05	2.69	4.00	1.79	1.79
262	AO	2.61	2.79		3.63	2.45								
1301	OA	2.28	2.50	2.04	2.85	1.87	2.51	2.11	2.20	1.40	2.19	3.22	2.45	2.78
243	AO	2.17	2.69	1.95	1.71	1.62	1.70	1.45	1.93	1.57	1.74	2.89	2.73	2.89

Figure 3. Copy number of loci in tumors as determined by QuMA. Forty-one cases of oligodendroglioma or oligoastrocytoma have been ordered by the extent of 1p loss beginning with tumors where all five 1p loci have been deleted. **Rows** represent individual cases, and copy numbers for genomic loci (position indicated in cM) have been arranged in **columns**. Pathological diagnosis is designated as: O, oligodendroglioma; AO, anaplastic oligodendroglioma; OA, oligoastrocytoma; AOA, anaplastic oligoastrocytoma. Gain or loss is assigned to a copy number by using a tolerance interval as previously described. For these data, a calculated copy number <1.58 was scored as a loss, whereas >2.53 was scored as a gain. Calculated numbers are as indicated in the **boxes**, and **dark-gray boxes** represent losses, **white boxes** normal, and **light gray boxes** indicate gains. Boxes without numbers represent loci not determined. Locations of the FISH probes relative to 1p and 19q markers are also indicated at the **top** of the diagram.

known to be lost at high frequency, was included as an internal control.²⁰ QuMA, as other molecular methods, detected frequent combined 1p/19q loss in oligodendroglial tumors of all grades. Interestingly, loss of two 10q markers was not observed in any tumors with whole arm loss of chromosome 1p/19q, whereas loss of a single 10q marker did occur in two cases. Specifically, 10q loss (both markers) was seen in 0 of 20 cases with whole arm 1p/19q loss, but 3 of 16 cases without whole arm 1p/19q loss. Although this was not quite statistically significant ($P = 0.078$, Fisher's exact test), these results are consis-

tent with previous data showing that these events do not generally occur concurrently.¹⁰

FISH

FISH analysis was performed with molecular probes from chromosomes 1p and 19q on a subset of paraffin-embedded tumors to test the accuracy of QuMA against an accepted molecular technique. An example of an oligodendroglioma showing loss of 1p with concordant results

Figure 4. Histology and molecular results from FISH and QuMA for oligodendroglioma. **A:** Histology of a typical oligodendroglioma (case 8758) showing round nuclei, perinuclear halos, and chicken-wire vasculature. **B:** FISH showing loss of 1p36 (red signal) relative to 1q24 (green signal). **C:** Delay of one cycle in a tumor DNA relative to a normal DNA at D1S468. PCR is performed in triplicate for both the microsatellite and reference loci for each DNA so that each curve actually represents three curves. Because the reference curves (six curves total) for the normal and tumor DNA reach threshold at precisely the same cycle, this experiment directly illustrates 1p loss through the delay of one cycle at the threshold in a tumor DNA relative to a normal diploid DNA.

by FISH and QuMA is shown in Figure 4. Copy number changes detected by QuMA and FISH were compared in 23 tumors (45 chromosomal arms) (Figure 5). Concordance between the two methods with respect to the scoring of loss or gain was evident at 37 of 45 chromosomal arms tested. We were primarily interested in the detection of 1p/19q deletions that may be indicative of responsive tumors. With respect to such deletions, FISH performed with a probe from 1p36 identified only a single deletion of chromosome 1p that QuMA did not detect (283). In this region of the chromosome, QuMA did, however, uncover a partial 1p deletion in one case (8503) that was not detectable by FISH (Figure 5). An additional case (14670) displayed a potential loss of the most distal marker (D1S468) by QuMA that was not concordant with FISH. In these cases, the deletions defined by the QuMA markers may not encompass the region containing the FISH probe. Overall, using FISH as a gold standard, the sensitivity for the detection of loss by QuMA for 1p and 19q was 92% and 94%, respectively. The specificity for

the detection of loss for 1p and 19q was 80% and 100%, respectively.

The identification of gains at 1p or 19q was not the focus of this study; however, in three cases (8503, 185, and 262), gains were detected by QuMA that were not evident by FISH. An additional case (14670) showed gain by FISH whereas QuMA detected no change in copy number at markers, D19S408 or D19S596, flanking the probe (Figure 5).

Molecular Diagnosis of Oligodendrogliomas

In general, gliomas that displayed many of the "classic" oligodendroglial features, such as round nuclei, perinuclear halos, and chicken-wire vasculature, tended to be those in which whole arm 1p/19q loss had occurred. There were, however, cases in which the molecular genetics provided useful or unexpected information. Examples of correlations of histopathology with molecular anal-

cM	FISH		QuMA		19q ratio	1p ratio	
	19q	1p	19q	1p			
8758	del	del	del	del	0.545	0.527	tetraploid
4541	del	del	del	del	0.571	0.530	
11654	del	del	del	del	0.565	0.605	
212	del	del	del	del	0.556	0.541	tetraploid
105	del	del	del	del	0.593	0.559	
2286	del	del	del	del	0.536	0.533	
1005	del	del	del	del	0.507	0.521	tetraploid
12730	del	del	del	del	0.525	0.526	tetraploid
12463	del	del	del	del	0.535	0.524	tetraploid
8282	del	del	del	del	0.521	0.516	tetraploid
6/SC	del	del	del	del	0.525	0.532	tetraploid
6829	del	del	del	del	0.577	0.582	
8503	del	nml	gain	del	0.766	1.000	
14670	gain	nml	nml	del	0.938	0.987	
191	del	nml	del	nml	0.541	1.002	tetraploid
195	del	nml	del	nml	0.618	1.000	
283	del	del	del	nml	0.533	0.543	tetraploid
48	nml	nml	nml	nml	0.993	0.992	
227	nml	nml	nml	nml	1.008	1.027	
236	nml	nml	nml	nml	0.986	0.995	
16223	nml	nml	nml	nml	0.979	1.010	
185	nml	nml	gain	gain	1.012	1.002	
262	nml	nml		gain	0.998	0.986	

Figure 5. QuMA and FISH results compared. FISH was performed on a subset of specimens from Figure 3 for comparison to QuMA results. Paraffin-embedded tumor sections were hybridized with probes for regions of loss on 1p and 19q. Hybridizations also included control probes from opposing arms, 1q and 19p, respectively, to create a ratio of total signals counted in 300 to 500 nuclei. Results are tabulated as nml for retention (two copies), del for loss (one copy), and gain for more than two copies. Chromosomal arms that were discordant between the two methods are shaded in the QuMA results.

ysis are shown in Figure 6. The histology of a classic oligodendroglioma (Figure 6A) with 1p/19q loss is contrasted with that of the tumor in Figure 6B. Although this tumor (2470) has some characteristics of anaplastic oligodendroglioma including a monomorphic appearance and lack of discrete tumor cell cytoplasm, it lacked classic features, such as round nuclei and perinuclear halos, but did show 1p/19q loss by QuMA. In Figure 6C, a

Figure 6. Correlation of 1p/19q status with histology. Photomicrographs (original magnification, $\times 400$) of H&E-stained paraffin sections from three oligodendrogliomas (A–C) and a single oligoastrocytoma (D and E) are shown. **A:** Case 5457: round nuclei and perinuclear halos highlight this classic oligodendroglioma with 1p/19q loss. **B:** Case 2470: an anaplastic oligodendroglioma that does not display classic oligodendroglial morphology, but showing 1p/19q loss. **C:** Case 16223: glioma with some features of oligodendroglioma (round, regular nuclei) where 1p/19q has remained intact. **D and E:** Case 2286: oligoastrocytoma with 1p/19q loss that exhibits distinct areas of oligodendroglial (D) and astrocytic (E) morphology.

tumor, diagnosed as oligodendroglioma on the basis of uniformly round nuclei, was found to have 1p/19q intact. Finally, six out of nine oligoastrocytomas had sustained loss of 1p/19q. Discrete regions of oligodendroglial and astrocytic morphology in one of these six cases is shown in Figure 6, D and E, respectively.

Minimally Deleted Region of 1p as Detected by QuMA

Chromosome 1p losses that accompany glial tumorigenesis are thought to signal the presence of a tumor suppressor gene(s). Many of the tumors with 1p loss in this study exhibited deletion of the entire chromosomal arm, but QuMA exposed partial deletions in four gliomas that may be useful in mapping the location of a putative tumor suppressor. In Figure 7, QuMA results define a common region of deletion from D1S468 to D1S2736 that is consistent with previous data from FISH and LOH analyses showing loss at 1p36.⁶

Discussion

The recent finding that 1p/19q loss was a marker of improved response to therapy and longer survival in patients with oligodendroglial tumors prompted our investigation into an assay for these markers that could be realistically applied both retrospectively (from archival material) and in a clinical setting for molecular diagnosis. With this in mind, we adapted QuMA,¹⁷ a novel real-time quantitative PCR assay, as a molecular diagnostic technique to rapidly assess 1p/19q status in oligodendrogliomas. In our survey of 41 gliomas with oligodendroglial features, whole arm 1p/19q loss with 10q retention emerged as a predominant genotype in both grade 2 and grade 3 tumors. FISH, using probes for 1p and 19q on 23 of these tumors, demonstrated concordance of findings with respect to the detection of 1p/19q loss. These results suggest that real-time quantitative PCR performed with a universal genomic probe may serve to supplement histology for clinical diagnosis of oligodendroglial tumors.

Our primary goal in this study was to develop a clinical assay to be used for the efficient detection of 1p/19q genetic losses in human oligodendrogliomas. Combined 1p/19q loss determined by QuMA was specific for tumors containing an oligodendroglial component as only a single astrocytic tumor displayed 1p loss (data not shown). Using FISH as the standard, QuMA was concordant at 37 of the 45 chromosomal arms tested. Only one case of 1p loss identified by FISH (283) was not detected by QuMA. As the FISH probe used in the study maps between the two telomeric QuMA markers used in this study (D1S214 and D1S468), it is possible that a small deletion may exist on chromosome 1p that was not identified by QuMA because of the location of our markers. However, the copy numbers determined by QuMA for these loci were 1.61 and 1.64, respectively, which was on the border of our cutoff for loss (1.58). Conversely, in two cases (14670 and 8503), small 1p deletions were identified by QuMA

Figure 7. 1p loss detected by QuMA maps to a common region of deletion previously characterized in gliomas. QuMA detected partial deletions (loss of more than one consecutive marker) in four oligodendrogliomas (8503, 307, 7876, 10896). All of these tumors have lost the marker D1S214. The FISH probe that is centromeric to D1S468 was retained in tumor 8503 limiting the overlapping region of loss from the probe region to the marker D1S2736. Tumor 14670 with an isolated loss at D1S468 was included to illustrate that retention of the FISH probe in both 14670 and 8503 raises the possibility that deletions in these tumors both end within the region complementary to the probe.

but not by the FISH probe used in this study. It is possible that the region of deletion in these two tumors does not include the region targeted by the FISH probe. Overall, the results indicated concordance between QuMA and FISH detection of loss at 1p and 19q. The fact that QuMA identified 10q loss as an infrequent occurrence with 1p/19q loss, consistent with other studies of oligodendroglioma, provides additional evidence that it is a valid technique to detect genetic loss.

In several cases, apparent gains in 1p or 19q were detected by either method but were not concordant. In three cases in which QuMA detected a gain that was not corroborated by FISH (8503, 185, and 262), 10 to 15% of nuclei in these specimens displayed gains by FISH but were not scored as a gain. Tumor heterogeneity may explain some of these discrepancies, although tumor DNA for QuMA and sections for FISH originated from the same paraffin blocks. In 8503, the primer sets used for QuMA may flank a small interstitial deletion that is detectable by the FISH probe. In one tumor (185), 9 of 11 loci across three chromosomal arms appeared to show gain by QuMA that was not corroborated by FISH. Because QuMA at a specific locus is dependent on comparison to a reference pool, it is possible that the reference pool does not represent a copy number of 2 in this tumor because of alterations sustained in the chromosomes where the reference pool primers reside. Copy numbers calculated using this pool as the reference locus could therefore be misleading in occasional cases.

A potential limitation of QuMA is the requirement for sufficient tumor DNA to perform the assay. As the assay is currently set up, no more than 15 ng of DNA is necessary for analysis per locus in addition to the reference pool. In our experience, obtaining sufficient DNA was a difficulty encountered primarily with older paraffin samples (>10 years), and the most recent cases (<2 years), easily yielded sufficient DNA quantities (μ g) of high quality (data not shown). This suggests that for some tumor samples, especially those obtained 10 years ago, and with small amounts of tumor, another method, such as FISH, may be more appropriate. However, little difficulty with respect to DNA isolation was encountered in the majority of recent cases. A second potential limitation of QuMA is contaminating genomic DNA from normal cells, which can dilute signal from tumor genomic DNA. It has been previously shown that loss can still be detected by QuMA in samples containing as much as 30% of normal

DNA.¹⁷ In addition, the use of microdissection, to increase the relative proportion of tumor cells from the paraffin section, can alleviate this problem in most cases.

Real-time PCR is dependent on copy number rather than polymorphism, and for this reason, all loci, theoretically, should be informative in each sample. However, under the standard cycling conditions, only some primer pairs will amplify with PCR efficiency close to 100% and yield a consistent δ Ct over a panel of normal DNAs. The standard deviations of the average δ Cts across the 10 normal DNAs did vary from 0.1 to 0.4, and those that yielded standard deviations >0.25 (or >0.5 of a copy), were unacceptable for analysis. In many of these cases, sufficient sequence was available so that alternative primer sets could be chosen for a locus. For example, microsatellite D19S596 was particularly important to study because it lies within a common region of loss on 19q defined by other laboratories.^{5,6,26-29} However, initial experiments indicated that the primer set described in the National Center for Biotechnology Information database was not suitable for QuMA (multiple bands appeared on amplification of genomic DNA indicating non-specific amplification). An alternative primer pair based on the known sequence flanking the CA repeat of this locus was designed that was ultimately successful for quantitation of copy number. This approach could be used for other repeats in regions of interest that do not amplify consistently in initial experiments.

QuMA, like FISH, will not detect genetic events that do not result in change in copy number, specifically loss of one allele followed by duplication of the remaining allele. Previous reports have demonstrated a high correlation between LOH and FISH on chromosome 1p in oligodendrogliomas indicating that for gliomas, in general, 1p deletion events will be assessed by methods that detect copy number.⁶ Primer sets may also lead to false conclusions about change in copy number in a specific tumor. For example, where polymorphisms or acquired mutations interfere with primer sets, loss may be thought to occur. Alternatively, a single locus may appear to be retained or amplified in contrast to copy numbers of flanking loci. For example, in two cases (4541 and 11654), D1S214 was retained whereas the other four markers, including one telomeric and three centromeric to D1S214, showed loss. For both of these tumors, analysis was performed at additional loci (12 total). QuMA detected loss at all of these markers, leaving D1S214 as

the sole retained marker in these tumors (data not shown). Several possibilities can explain these data. First, the region encompassed by D1S214 is retained in these tumors. Second, normal alleles for a specific locus may exist in the human population in which some feature of the allele (size of the repeat element, for example) may affect amplification to an extent that the resultant δCt deviates significantly from the average of a panel of normals. In essence, this repeat would represent a non-informative locus in the analysis of the corresponding tumors. Third, a genetic event that resulted in a small region of gain has occurred at this site, and it appears to be retention. Finally, because the TI was calculated with 95% confidence, we expect that 5% of the numbers may be scored incorrectly. An advantage of QuMA is that many loci can be analyzed rapidly and efficiently along a chromosomal arm such that, similar to LOH analyses, divergent copy numbers require evaluation in the context of data from flanking markers.

This type of genetic analysis may be particularly useful for clinical cases in which the histological features are ambiguous. Neuropathologists often differ with respect to the distinction of astrocytomas and oligodendrogliomas. Complicating matters are the oligoastrocytomas, which are a mixed subtype of glioma that histologically exhibit both oligodendroglial and astrocytic features. Some cases of oligoastrocytoma contain well-defined alternating sections of oligodendroglioma or astrocytoma within a single tumor. Although demarcated regions may exist within a tumor, these cases have been shown to still be genotypically clonal and furthermore, they either display genetic alterations typical of one or the other, but never both.^{30,31} For example, mutation of p53 is common in astrocytic tumors but rarely occurs in oligodendrogliomas. In oligoastrocytomas where 1p/19q loss has occurred, p53 mutation, as in oligodendrogliomas, is not generally observed.³¹ Nine of the tumors analyzed were diagnosed as mixed oligoastrocytomas (both grade 2 and grade 3), and in six of these cases, 1p/19q loss was found. One of the oligoastrocytomas, tumor 48, was diploid at most loci tested and in this respect, was genotypically similar to tumors of astrocytic lineage. Interestingly, this tumor exhibited positive immunohistochemical staining for p53 which often represents mutation of the gene.³²

In addition to the clinical ramifications of QuMA, this technique may facilitate the identification of tumor suppressor genes that may be the targets of recurring 1p and 19q losses in oligodendrogliomas.^{4,6,26-29,33} QuMA, like LOH, has the resolving power to detect small deletions, including homozygous deletions,¹⁷ and may help in mapping the location of the putative tumor suppressors. Partial deletions of 1p (loss of more than one consecutive marker) were detected in four gliomas. The smallest common region of deletion is defined by markers D1S468 and D1S2736 (Figure 7). An additional tumor, 14670, showed a possible loss of the most distal marker, D1S468. These data, in the context of the FISH results, raise two interesting possibilities regarding the location of a putative tumor suppressor gene(s). The first is that completely nonoverlapping deletions exist between 14670 and 8503.

The FISH probe lies between markers D1S214 and D1S468, and in both tumors, the probe was retained. Therefore, the region complementary to the FISH probe may not be contained within the minimal region of deletion for either tumor. The second possibility is that the deletions in 14670 and 8503 each end within the BAC used for FISH. In this scenario, hybridization by the BAC is still possible because sufficient sequence that is complementary to the probe is retained in each tumor. Finally, tumor 283 exhibited a partial deletion by QuMA that did not overlap with regions of loss in the other four tumors (Figure 3). Nonoverlapping regions of 1p loss have also been observed in another series of oligodendroglial tumors.³³

The ease of QuMA makes it feasible to survey a tumor genome in a clinical setting in a rapid and efficient manner using paraffin material obtained from the pathology laboratory. It is hoped that this technique may be applied to a number of tumor types to supplement histological diagnosis to refine prognostic and therapeutic decisions for specific patients.

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