Glioma Test Array for Use with Formalin-Fixed, Paraffin-Embedded Tissue

Array Comparative Genomic Hybridization Correlates with Loss of Heterozygosity and Fluorescence *in Situ* Hybridization

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Array-based comparative genomic hybridization (aCGH) is a powerful, high-throughput tool for whole genome analysis. Until recently, aCGH could only be reproducibly performed on frozen tissue samples and with significant tissue amounts. For brain tumors however, paraffin-embedded tissue blocks from small stereotactic biopsies may be the only tissue routinely available. The development of methods to analyze formalin-fixed, paraffin-embedded (FFPE) material therefore has the potential to impact molecular diagnosis in a significant way. To this end, we constructed a BAC array representing chromosomes 1, 7, 19, and X because 1p/19q deletion and *EGFR* gene amplification provide clinically relevant information for glioma diagnosis. We also optimized a two-step labeling procedure using an amine-modified nucleotide for generating aCGH probes. Using this approach, we analyzed a series of 28 FFPE oligodendroglial tumors for alterations of chromosomes 1, 7, and 19. We also independently assayed these tumors for 1p/19q deletion by fluorescence *in situ* hybridization and by loss of heterozygosity analyses. The concordance between aCGH, standard loss of heterozygosity and fluorescence *in situ* hybridization was nearly 100% for the chromosomes analyzed. These results suggest that aCGH could offer an improved molecular diagnostic approach for gliomas because of its ability to detect clinically relevant molecular alterations in small FFPE specimens. (J Mol Diagn 2006, 8:268–276; DOI: 10.2353/jmoldx.2006.050109)

dendrogliomas are distinguished by their sometimes remarkable sensitivity to chemotherapy. Allelic loss of chromosome 1p is a strong predictor of chemosensitivity, and combined loss of chromosome 1p and 19q is statistically significantly associated with both chemosensitivity and longer recurrence-free survival after chemotherapy.¹ Loss of 1p also appears to have prognostic importance in low-grade oligodendrogliomas.^{2,6} Taking clinical and molecular heterogeneity into consideration,⁷ anaplastic oligodendrogliomas can be divided into different genetic subgroups.⁸ One subgroup represents patients whose tumors have combined but isolated losses of 1p and 19q. This group shows marked and durable responses to chemotherapy associated with longer survival, with or without postoperative radiation therapy. Another subgroup includes tumors without 1p loss, which are associated with more aggressive behavior. Thus, genetic analysis can be used to tailor therapy at the time of diagnosis. This work has sparked considerable interest in molecular markers for glioma management and has prompted diagnostic 1p/19q testing in many research centers worldwide.

There are two methods routinely used by most laboratories to assess 1p/19q loss: loss of heterozygosity (LOH) analysis using microsatellite markers and fluorescence *in situ* hybridization (FISH) analysis. Both methods have several advantages and disadvantages. Because LOH is a polymerase chain reaction (PCR)-based method, multiple markers can be screened at one time, but expanding the test to many markers may be expensive. In addition, with the LOH approach, the need for constitutional DNA for comparison with tumor DNA usually requires a blood sample. This has been a major hindrance for LOH testing and has led to the introduction of FISH for 1p/19q testing as a preferred method. FISH analysis does not require constitutional DNA, but the technique has other disadvantages. FISH testing for multiple loci requires

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Classification of human gliomas based on molecular genetic alterations has already achieved clinical relevance.^{1–6} Among the major subtypes of gliomas, oligo-

optimization of test and control probes for each locus, which can be laborious. Scoring large numbers of cells to get statistically relevant results is also time consuming and therefore expensive.

The ability to detect a wide variety and potentially large number of genetic changes will be important for future molecular diagnostic applications. In array comparative genomic hybridization (aCGH), arrayed DNA targets are co-hybridized with differentially labeled tumor and reference DNAs. DNA copy number along the genome is proportional to the test:reference ratio of fluorescence intensities. Array CGH thus enables screening of large representations of the human genome and offers the additional advantages of increased sensitivity for smaller genomic regions and increased sensitivity for detecting subtle copy number differences.^{9–14} Problematically, however, the signal-to-noise ratio may be quite low when DNA isolated from formalin-fixed tissue is used for aCGH analysis, which compromises the ability to use aCGH as a diagnostic tool to study small, fixed specimens.

We report the feasibility of performing aCGH using DNA from archival, formalin-fixed, paraffin-embedded (FFPE) human oligodendroglioma samples and a labeling procedure to generate high-quality probes for array hybridization. We demonstrate this technique using a proof-of-principle BAC array representing sample chromosomes 1, 7, 19, and X and compare the detection of chromosomal copy number changes with standard LOH and FISH assays.

Materials and Methods

Tissue and DNA Samples

FFPE tissue blocks for 32 gliomas, as well as frozen tumor tissue for 4 gliomas, were obtained from the Massachusetts General Hospital after appropriate human studies approval. The tumor samples were less than 5 years in age (1999 to 2004). All of the glioma samples had distinct oligodendroglial features on histopathological examination. The tumor panel included 14 grade II oligodendrogliomas, 9 anaplastic oligodendrogliomas, 1 grade II oligoastrocytoma, and 8 anaplastic oligoastrocytomas. Tumor DNA was extracted from four 6- μ m-thick unstained sections adjacent to a hematoxylin and eosin (H&E)-stained section, following a standard protocol.¹⁵

Array Construction

A BAC array was constructed containing 200 targets that represented chromosomes 1, 7, 19, and X. BAC clones were purchased from Invitrogen Corp., Carlsbad, CA. We included 100 BACs for chromosome 1, 12 BACs including the *EGFR* region for chromosome 7, 50 BACs for chromosome 19, 30 BACs for chromosome X, total human genomic DNA as a positive control, and λ DNA as a negative control. DNA from the same BAC clones used for 1p and 19q FISH and BACs mapping to the close proximity of the LOH markers were included in the array. Each clone was screened by PCR using a unique se-

quence-tagged site or expressed sequence tag marker corresponding to each BAC clone. DNA from PCR-positive BAC clones was prepared using the Qiagen plasmid DNA isolation kit (Qiagen Inc., Valencia, CA). DNA quality was tested by performing an *EcoRI* digest, and each clone was mapped by FISH. Only PCR-confirmed BACs that mapped accurately were included in the array. Targets were generated by degenerate oligonucleotide-primed PCR using a degenerate primer with an amine linked to the 5'-end. Target solutions were prepared by resuspending DNA at a concentration of 250 ng/ μ l in 150 mmol/L sodium phosphate buffer, pH 8.0. Targets were printed in quadruplicate on three-dimensional-link slides (Amersham Biosciences Corp, Piscataway, NJ) using an OmniGrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI). After printing, arrays were incubated for 16 to 18 hours at 100% relative humidity to allow amino-linked DNA to bind covalently to the surface. Arrays were then postprocessed to block all unreacted sites with glycine. To quality control the spot morphology, a sample array was hybridized with Alexa 532-labeled random 9-mer oligodeoxynucleotide (Molecular Probes, Eugene, OR).

DNA Labeling

One μ g of each frozen and FFPE tumor, and of reference genomic DNA, were labeled by random priming using a Bioprime labeling kit (Invitrogen).¹⁶ A two-step labeling protocol using amino-allyl-dUTP for labeling FFPE DNA was also optimized. For the amino-allyl labeling method, an amine-modified nucleotide (aadUTP) was first enzymatically incorporated into test and reference genomic DNAs. The amine-modified DNA was then chemically labeled using an amine-reactive fluorescent dye. Briefly, 1 μ g each of test or reference DNA was combined with 10 μ g of random hexamers and water to 42 μ l, incubated at 100°C for 10 minutes, and chilled on ice for 5 minutes. To each reaction, 5 μ l of 10 $\times$ Klenow buffer (0.5 mol/L Tris-HCl, pH 7.5, 100 mmol/L MgCl₂, 10 mmol/L dithiothreitol, 0.5 mg/ml bovine serum albumin) 2 μ l of dNTP mix (3 mmol/L dATP, dGTP, dCTP, 1.2 mmol/L dTTP, and 1.8 mmol/L of aadUTP), and 40 U of Klenow polymerase were added on ice. The reactions were then incubated at 37°C for 2 hours. Labeled DNAs were purified using Qiagen PCR purification columns. Purified DNAs were eluted in 50 μ l of nuclease-free water and then chemically labeled with Alexa Fluor 555 and 647 (Molecular Probes) for 2 hours at room temperature in the dark. Free dyes were quenched by adding 15 μ l of 4 mol/L hydroxylamine to each reaction, and reactions were incubated at room temperature for 15 more minutes. Reactions were then purified using Qiagen PCR purification columns and used for array hybridization.

Array Comparative Genomic Hybridization

Labeled test and reference DNA samples were mixed with 100 μ g of Cot-1 DNA and ethanol precipitated, then resuspended in 20 μ l of hybridization solution containing 50% formamide, 10% dextran sulfate, 2 $\times$ standard saline

citrate, 2% sodium dodecyl sulfate, and 100 μ g of yeast tRNA. The probes were denatured for 5 minutes at 72°C and then preannealed for 1 hour at 37°C before hybridization. Arrays were placed in prewarmed airtight hybridization chambers humidified with 2 $\times$ standard saline citrate and 0.1% sodium dodecyl sulfate to prevent evaporation, and the probe mixture was hybridized for 48 hours under a lifter slip (Erie Scientific, Portsmouth, NH) at 37°C. Slides were washed for 15 minutes with 50% formamide, 2 $\times$ standard saline citrate, pH 7.0, 0.1% sodium dodecyl sulfate at 50°C, and once in 0.1 mol/L sodium phosphate buffer, 0.1% Nonidet P-40, pH 8.0, for 10 minutes at room temperature. Finally the slides were rinsed in distilled water and spun down at 1000 rpm for 2 minutes to dry.

Data Analysis

Arrays were scanned with an Axon 4000B scanner, and images were analyzed using GenePix Pro 4.1 and Acuity software (Axon Instruments, Inc., Union City, CA). Spot exclusion criteria included removal of spots with uneven hybridization signals or close proximity to nonspecific background noise. A series of five normal versus normal hybridizations were performed to define the normal variation of the test to reference log₂ intensity ratio for each target clone. The average log₂ ratio of medians of test to reference was used for statistical analysis.

Statistical Analysis

The log₂ ratios for a given hybridization were normalized by centering them at the median value of the log₂ ratios at chromosome 1q for that hybridization.¹⁷ The log₂ ratios on 1q were chosen for normalization rather than the entire set of log₂ ratios because of the lack of loss and gain expected on 1q (which was confirmed on FISH analysis) and the high frequency of loss expected on the majority of the remaining BACS on the array (ie, those that reside on 1p and 19q).¹⁷ Binary segmentation algorithm¹⁸ was then applied to detect change points along the chromosomes that define segments of loss and gain. Segments were considered to represent true losses or gains according to whether their associated absolute mean log₂ ratio levels were greater than

$$2 \times \sigma / \sqrt{n},$$

With σ estimated to be 0.58, the empirical estimate of the SD of the standardized segment means for 1q, and n equal to the number of clones in the given segment. For example, a 19q segment that contains 26 clones was considered to represent loss if its estimated mean level was less than -0.23 . A 1p segment that contains 57 clones was considered to represent loss if its estimated mean level was less than -0.16 . A 1p segment that contains 30 clones was considered to represent loss if its estimated mean level was less than -0.22 .

Figure 1. Array validation. aCGH was performed using male and female lymphocyte DNA from healthy donors. Male DNA was labeled with Cy3-dCTP and female DNA was labeled with Cy5-dCTP by random priming. Hybridization was performed mismatching the sex chromosome. Log₂ ratios along the autosomes appear normal and along the X chromosome appear deleted, indicating the sex mismatch for the X chromosome. Ratio profiles from five different control hybridizations are shown. **Arrows** indicate centromeres of 1 and 19.

LOH Analysis of 1p/19q Regions

PCR-based LOH analysis was performed using microsatellite markers D1S508, D1S199, and D1S2734 for 1p and D19S219, D19S112, and D19S412 for 19q.¹⁹ LOH was performed on the same DNA that was used for aCGH.

FISH Analysis for 1p/19q Deletions

Dual-color FISH was performed on tissue sections using BACs mapping to 1p36.2/1q21 (RP11-558F24 and RP11-459O3) and BACs mapping to 19q13.3/19p13.3 (RP11-293G10 and RP11-75H6), following a published protocol.²⁰ At least 200 cells were scored for each probe set. Relative copy numbers for 1p/1q and 19q/19p were counted, and a ratio of 0.7 or less for 1p:1q and/or 19q:19p was considered a loss. We chose a relatively stringent cutoff ratio of 0.7 to evaluate the sensitivity of aCGH for detecting definitive loss in paraffin DNA. The goal was to have samples with clear loss by FISH to help interpret the aCGH result accurately.

Results

Array Validation

The sensitivity of the array was tested by hybridizing normal human male and female lymphocyte DNAs labeled by random priming with Cy3- and Cy5-dCTP, respectively. The normal versus normal hybridizations were repeated five times. All five gender-mismatched normal versus normal hybridizations showed log₂ ratios close to 0 along the autosomes and a decrease or increase of X chromosome copy number as expected by the gender mismatch (Figure 1).

Comparison of Labeling Procedures

FFPE DNA from six oligodendrogliomas was used to compare results from amino-allyl-labeled DNA against those obtained using random primed DNA probes. DNA from each tumor sample was labeled both by random priming and by amino-allyl-dUTP and hybridized to the

Figure 2. Comparison of labeling procedures. DNA from oligodendroglioma blocks was isolated as described in the Materials and Methods section. **a** and **b**: A tumor without 1p/19q loss, with **a** labeled with random priming and **b** labeled with amino-allyl-dUTP; sex is mismatched. **c** and **d**: A tumor with 1p/19q loss, with **c** labeled with random priming and **d** labeled with amino-allyl-dUTP; sex is mismatched. In both cases, the amino-allyl-dUTP-labeling procedure produced smoother ratio profiles compared to randomly primed labeled DNA. **Arrows** indicate centromeres of 1 and 19.

array. Gender was mismatched between the test and reference probes to use the X chromosome BACs as an internal control. Of the six tumors tested, five had 1p/19q loss and one had both copies of 1p/19q. In all cases with 1p/19q loss, the copy number change was detected by both labeling methods, but the random-primed probes produced greater variability compared to the amino-allyl-dUTP probes. The ratio profiles of two tumors, one with and one without 1p/19q loss, are shown in Figure 2, a–d. Given the reduced variability noted with amino-allyl-dUTP labeling, this method was used for evaluation of the larger panel of tumors.

Comparison of Frozen Versus FFPE DNA

Four oligodendrogliomas having both frozen as well as FFPE tissues were studied to compare results between frozen and FFPE materials. DNA from frozen samples was labeled by random priming, and the ratio profiles showed two of these tumors to have 1p and 19q deletions. DNA from the FFPE tissues from these two samples was labeled by amino-allyl-dUTP and the data were compared with the random priming-labeled DNA from the frozen tissues (Figure 3, a–d). In both tumors, results from frozen DNA labeled by random priming and FFPE DNA labeled by amino-allyl-dUTP were similar; both detected losses at 1p and 19q and no changes at 1q, 7, and 19p. Even though FFPE DNA had greater variability, the overall patterns of loss detected by both labeling procedures were the same.

Overall Comparison of aCGH, FISH, and LOH

aCGH, LOH, and FISH for 1p and 19q were performed on a panel of 28 oligodendroglial tumors. Of the 28 tumors, 16 had loss of 1p and 19q and 5 had intact 1p and 19q, as detected by all three methods (eg, Figure 4, a and b). Two tumors had intact 1p but deleted 19q, as shown by all three methods. Thus, there was complete concordance between the three methods in 23 of the 28 cases (82%), leaving 5 with discrepancies (Table 1). Of note, amplification of the *EGFR* gene was found in one tumor by aCGH and was subsequently confirmed by FISH (Figure 4d).

Discordant Cases

Case 1 was discordant at 19q, with loss detected by LOH and aCGH but not by FISH. Initial FISH scoring showed multiple populations of cells: 49% of cells had equal copies for both 19p and 19q; and the remaining 51% of the cells had copy numbers ranging from two to four for 19p and one to three for 19q, indicating relative loss of 19q in these cells. Because there was a high degree of aneusomy present for chromosome 19 in this tumor, the FISH ratio of 19q:19p signals for all cells combined was 0.785, which was close to being scored a loss but did not make the formal cutoff ratio of 0.7. (Of note, if we had used a less stringent cutoff ratio of 0.8, FISH would have agreed with aCGH and LOH in this case.) Loss of 19q was detected both by aCGH and LOH analysis, including one repeat LOH study. Thus, the discordant results arose because of difficulty scoring this aneusomic case on FISH.

Figure 3. Comparison of frozen versus FFPE DNA in two oligodendrogliomas. Array CGH was performed using frozen DNA (**a** and **b**) labeled by random priming. 1p and 19q loss was clearly visible in both samples. Sex chromosomes were mismatched as an internal hybridization control. FFPE DNA from the same tumors labeled by amino-allyl-dUTP clearly detected the losses, as shown in **c** and **d**. Arrows indicate centromeres of 1 and 19.

Case 2 was discordant at 1p, with deletion detected by LOH and aCGH but not by FISH. Initial FISH analysis showed 50% of cells with two copies of 1p and 1q, 35% of cells with three copies of 1p and q and the remaining 15% showed loss of 1p. To further evaluate the discordance, FISH and LOH was repeated using serial sections from the same block of tissue. The repeat FISH score showed a combined ratio of 0.69 for 1p and 0.67 for 19q, indicating loss. LOH analysis of the same DNA also showed clear loss of both 1p and 19q markers. In re-evaluating the scoring differences between the two FISH assays, it was noted that this tumor had excessive neuropil causing high background autofluorescence, which likely interfered with signal quantification.

Case 3 was discordant at 19q, with loss detected by LOH and aCGH but not by FISH. Initial FISH analysis showed 10% of cells with loss of 19q and 90% of cells with two copies of 19p and 19q. To evaluate the discrepancy, both FISH and LOH were performed on the same areas of a different block containing solid tumor. The repeat FISH scoring showed 20% of cells with one copy of 19p and 19q, 51% with two copies of 19p and one copy of 19q, and the remaining 29% with two copies of both 19p and 19q signals. The 19q:19p ratio of 0.7 was consistent with loss of 19q. LOH analysis was performed using DNA from the same tissue and the result showed loss of 19q markers. In comparing the FISH results from both assays, there were no technical differences in the quality of the two runs, but it was noted that the initial FISH and second FISH were from different regions of the tumor, with the second FISH results deriving from the same tissue also studied for LOH. Thus, intratumoral heterogeneity resulted in sampling error, accounting for different FISH results.

Case 4 was the most difficult to resolve, because 1p loss was noted only with aCGH and 19q loss was not detected by LOH analysis. Initial FISH analysis with the 1p probe set showed multiple populations of cells: 71% of cells had two copies of both 1p and 1q, 15% of cells had loss of 1p and the remaining 14% had three to four copies of both 1p and 1q signals. For 19q probe set, 80% of cells had one copy of 19q, 13% of cells had two copies of 19p and 19q, and the remaining 7% of cells had three copies of 19p and 19q signals. Loss of 1p and 19q were detected by aCGH. LOH did not detect a loss of either 1p or 19q. For this case, FISH was repeated for 1p and showed three populations of cells with one to three copies of 1p signals, with more than 75% of cells having loss of 1p. These cells were confined to a very small region of the tissue section. In addition, the original LOH gels were reviewed by an independent reviewer; although both alleles were present, the reviewer felt that all markers showed allelic imbalance, suggesting the presence of a population of cells with LOH and considerable contamination by cells maintaining both copies. In summary, there was intratumoral heterogeneity resulting in sampling error (as in case 3) as well as difficulty scoring LOH results. Finally, in case 5, FISH detected increase in copy number for both 1p and 1q probes in ~50% of the cells scored, aCGH detected gain of chromosome 1, but LOH did not detect the gain (Figure 4c).

Discussion

The goals of this study were to test the feasibility of using DNA from FFPE glioma samples for aCGH and to obtain preliminary estimates of the diagnostic correlations be-

Figure 4. Comparison of aCGH, FISH, and LOH in representative oligodendroglial samples. **a:** aCGH of xT-3608 shows normal 1p and 19q copy number. aCGH result was confirmed by FISH and LOH using markers described in the Materials and Methods. **b:** aCGH of xT-3533 shows loss of 1p and 19q. aCGH result was confirmed by FISH and LOH. **c:** aCGH of xT-3643 shows increase in copy number for chromosome 1. FISH analysis on tissue section shows increase in copy number for both 1p and 1q probes. **d:** aCGH of xT-3508 shows amplification of the *EGFR* gene. Three additional clones from 7p mapping in close proximity to the *EGFR* gene and five clones from 7q were included in the array. None of the other chromosome 7 clones were increased in copy number or amplified. aCGH result was confirmed by FISH on tumor section. The sex chromosomes were mismatched for all hybridizations. Red "lollipop" denotes the BAC clone used for FISH and green arrows denote positions of markers used for LOH analysis relative to the BAC used for FISH. Downward arrows indicate centromeres of 1 and 19.

tween aCGH, FISH, and LOH for detecting 1p and 19q loss in gliomas. Using tumor samples from the last 5 years (1999 to 2004), we performed aCGH, FISH, and LOH in a panel of 28 archival oligodendroglial tumors and

compared the results of these three methods. We conclude that aCGH could be useful as a diagnostic tool to assess DNA copy number changes in FFPE glioma samples. It may also prove useful as a research tool to de-

Table 1. Summary of aCGH, LOH, and FISH Comparison in the Discordant Cases

Case no.	Diagnosis	1p aCGH	19q aCGH	1p LOH	19q LOH	1p FISH	19q FISH	Explanation
1	AOA III	N	L	1,2	LOH (LOH)	MAIN	MAIN (LOSS)	FISH scoring complicated by aneuploidy
2	AOA III	L	L	LOH	LOH	MAIN (LOSS)	LOSS	FISH scoring complicated by aneuploidy and autofluorescence of neuropil
3	AOA III	N	L	1,2	LOH	MAIN	MAIN (LOSS)	FISH scoring complicated by sampling error and heterogeneity
4	AO III	L	L	1,2 (AI)	1,2 (AI)	MAIN (LOSS)	LOSS	FISH scoring complicated by sampling error and heterogeneity; LOH scoring difficult secondary to allelic ratios
5	AOA III	G	N	1,2 (GND)	1,2	TRI	MAIN	LOH unable to detect copy number gain

Discordant results are shown in bold. Results from the repeat experiments are given in parentheses. AOA, anaplastic oligoastrocytoma; AO, anaplastic oligodendroglioma; N, two copies detected by aCGH; L, one copy detected by aCGH; G, gain detected by aCGH; 1,2 normal, informative; LOH, loss of heterozygosity; AI, allelic imbalance; GND, gain not detected; MAIN, maintenance of both copies by FISH; LOSS, loss of one copy by FISH; TRI, trisomy.

termine DNA copy number changes of prognostic or predictive significance, because many study cases may also only be available for research purposes as FFPE samples.

The utility of aCGH to identify increases and decreases of DNA copy number was first reported by Pinkel's group.⁹ Using a high-density array for chromosome 22, aCGH has also been shown to detect heterozygous and homozygous deletions at the NF2 locus.¹⁴ In addition, aCGH has been used to identify gene amplifications in glioblastomas and breast cancers.^{21,22} However, most studies that have shown that aCGH can be used to quantitate chromosomal copy number in human and mouse tumors have used fresh, frozen tissue.

To our knowledge, only three previous reports have commented on the use of FFPE DNA for aCGH.^{16,22,23} Daigo and colleagues²² used FFPE DNA amplified by degenerate oligonucleotide-primed (DOP) PCR and an array consisting of oncogenes. Unfortunately, this array is not as relevant for detecting DNA copy number loss (given the emphasis on oncogene loci), which is of importance for glioma molecular diagnostics. Paris and colleagues¹⁶ used FFPE DNA from archival prostate tumor samples and a ~1.4-Mb resolution array representing the human genome. This group, however, did not amplify tumor DNA and labeled DNA by random priming. They analyzed 20 prostate cancer samples both by aCGH and conventional chromosomal CGH. For two tumors, CGH showed loss of 13q21, but aCGH did not detect it, which may have been related to the size of the deletions and the resolution of the arrays. These authors reported loss and gain of single BAC clones mapping to hundreds of loci throughout the genome, but the results were not confirmed by FISH, and wide spacing between BACs make interpretation of losses and gains difficult. DeVries and colleagues²³ compared DOP amplification and random primed amplification, a procedure routinely used in many laboratories for labeling genomic as well as cDNA samples, in FFPE breast cancer tissues. These

authors reported that one additional round of random primed amplification, using 5 to 50 ng of starting DNA, yielded sufficient DNA for hybridization. Notably, however, the authors used an amplicon on 17q to compare the sensitivity of aCGH between fresh frozen and FFPE DNA. In our experience, large amplicons such as these are easily detectable using DNA from either frozen or fixed tissues. In our array, for example, we included four BAC clones containing the *EGFR* gene. All four clones produced almost identical log2 ratios in the tumor with *EGFR* amplification (Figure 4d).

Given that chromosomal losses typically represent a decrease of only one copy number from normal, detecting loss of DNA copy number can be more challenging than detecting amplification, particularly when using FFPE DNA. DeVries and colleagues²³ showed significant reduction in the noise level when a random primed amplification-generated DNA probe was used rather than a DOP-amplified DNA probe. We also found that DOP amplified DNA from FFPE tissue, when used as probe for aCGH, produced very low signal-to-noise ratio (data not shown). We therefore decided to label DNA without amplification using a different labeling protocol. For this reason, we optimized an amino-allyl-dUTP-labeling procedure for FFPE DNA. In this procedure, FFPE DNA is first enzymatically labeled with amino-allyl-dUTP, and the modified DNA is then chemically labeled with an amine reactive dye. This labeling procedure proved superior to random priming for labeling FFPE DNA and did not introduce any bias whether DNA was labeled with Cy3- or Cy5-dCTP. This produces homogeneous hybridization signal and significantly reduces the background.

In the initial analysis of our tumor panel, 23 of the 28 tumors showed identical results between the three procedures used to detect single copy loss in FFPE gliomas. The discordant results in five cases beg the question of which method should be considered a gold standard. aCGH is the newcomer to the field, but to date there is no consensus on whether LOH or FISH is the gold stan-

dard.²⁴ The two original reports documenting genetic correlations with chemotherapeutic response in anaplastic oligodendrogliomas^{1,8} and a recent study showing an association between chemotherapeutic response and 1p status in low-grade oligodendrogliomas²⁵ used LOH analysis. On the other hand, studies that have correlated 1p/19q status with overall survival (ie, not with chemotherapeutic response)⁴ have used FISH, and FISH has certainly become the more widespread test performed in North America, primarily because of its relative ease for pathology departments.²⁴ At the present time, therefore, one must recognize that LOH has been the most popular method relative to predicting chemotherapeutic response, but it remains possible that both techniques provide similar prognostic and predictive power. Comparisons have been made previously for FISH, LOH, and standard CGH. For example, the careful study of Smith and colleagues²⁶ found correlation coefficients for detection of 1p and 19q alterations of 0.98 and 0.87 for LOH and FISH, respectively, 0.79 and 0.60 for LOH and standard CGH, and 0.79 and 0.53 for FISH and standard CGH. These results suggest excellent concordance for 1p assessment between LOH and FISH, less close correlation for 19q status, and not surprisingly, decreased sensitivity for standard CGH. Clearly, however, there are individual cases that do not yield the same result with the two techniques. In this regard, it must be recognized that, without comparison against chemotherapeutic response status, one cannot conclude which of the two techniques is a better clinical predictor.

In the initial analysis from our study, five tumors did not show absolutely concordant results for both chromosome arms. On further consideration or additional work-up, these apparently discordant results can be explained. Four of the five cases (Table 1, cases 1 to 4) were discordant as a result of difficulties with scoring FISH assays. In case 1, aneusomy complicated scoring; for those laboratories using FISH analysis, in this regard, the setting of aneusomy and a score close to the cutoff for calling a loss should perhaps prompt analysis of the case with one of the other methods. Case 2 was also aneusomic, with scoring additionally complicated by the tumor being highly infiltrative of the adjacent brain; such tumors can be difficult to count when autofluorescence of the background neuropil obscures the cells and FISH signals. In this situation, there is a tendency to score small islands of cells away from the areas with extensive entrapped neuropil. Consequently, there appeared to be a bias toward selecting areas of the tumor with good hybridization and less autofluorescence. In case 3, regional variability, with the deletion confined to a region of the tumor, proved more of a problem with FISH than with the more regionally averaging LOH and aCGH approaches. In cases 2 and 3, which were both infiltrative cases, FISH also showed a propensity to underscore loss, which was presumably overcome by the wider sampling of tumor cells by aCGH and LOH. Because of these problems, there was also considerable interobserver variability in FISH scoring of these cases.

In the fourth discordant tumor, FISH scoring was complicated by regional variability, again illustrating

the problem of potential sampling bias. The case also highlighted some problems with scoring LOH assays. Review of LOH results for both 1p and 19q markers showed some allelic imbalance that was not distinct enough to be scored as a loss on initial review. The subjective scoring criteria used for LOH is a potential problem resulting in false-negatives. Quantitative approaches may obviate some of these problems, but setting of cutoff values can be difficult nonetheless. One of the major problems with molecular diagnosis in gliomas is the fact that these tumors are infiltrative of the normal brain; as a result, most specimens have a moderate admixture of nonneoplastic cells. Sensitivity to detection of single copy number changes in neoplastic cells can therefore be a major challenge. This is partially compensated for by careful preselection of regions for DNA extraction and for FISH analysis based on histological examination of an adjacent H&E-stained section. However, some of the tumors are highly infiltrative, and histological guidance may not help select a more solid area. In this not uncommon situation, as was seen in this last case, having a more sensitive molecular assay for finding single copy loss would offer a major benefit. The results in this last case suggest that aCGH, by providing an inherently more quantitative method, may also provide a more sensitive assay for detecting single copy losses. Although not evaluated in quantitative detail in this panel, it was our impression that aCGH and LOH detected loss when ~50% of cells had loss by FISH.

Case 5 is not truly discordant but simply reflects the inability of LOH assays to detect chromosomal gains. Clearly, aCGH and FISH are superior methods compared to LOH for detecting copy number gains. Of note, copy number gain, particularly at the *EGFR* gene, may be of prognostic and classification significance in gliomas. For instance, knowledge of *EGFR* gene amplification may be useful in assessing prognosis in anaplastic oligodendrogliomas¹ and in subtyping glioblastomas.²⁷ For these reasons, an assay that can measure both chromosomal gain and loss offers distinct advantages. As a result, approaches such as aCGH and FISH are preferable to LOH if chromosomal gains and gene amplification are to be evaluated alongside losses. This is likely to be the case with human gliomas.

In summary, we have demonstrated a novel labeling technique that enables aCGH to be performed using DNA from FFPE tissues. In addition, we have shown that aCGH provides similar (but not identical) results to the standard assays of LOH and FISH in assessing clinically relevant single copy losses, and we have raised the possibility that aCGH may offer advantages over these other techniques in certain situations. In the future, if molecular diagnostic approaches to gliomas necessitate analysis at multiple genetic loci, aCGH will also offer the advantage of ready scalability. At the present time, aCGH is more costly and less widely available than LOH and FISH technologies; however, it is likely that aCGH costs will decrease and availability will increase as the utility of this approach broadens in the next few years. We conclude that FFPE DNA from glioma samples can be used

for detection of single and multiple copy number DNA losses and gains by aCGH and suggest that aCGH may prove to be a useful tool for high-throughput diagnostic testing in the not too distant future.

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