Characterization of the 1p/19q Chromosomal Loss in Oligodendrogliomas Using Comparative Genomic Hybridization Arrays (CGHa)

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Abstract. Loss of genetic material from the short arm of chromosome 1 and the long arm of chromosome 19 in anaplastic oligodendrogliomas has been shown to predict responsiveness to chemotherapy. Currently, the most common approach used to detect this loss of 1p/19q material employs microsatellite/FISH analysis using markers along the length of these chromosome arms. This analysis is highly focused and carried out on a locus-by-locus basis and gives no indication of the extent of other genetic changes occurring in the tumor cells, which may be important in future studies to explore genetic heterogeneity in the response to treatment. We have investigated the use of comparative genomic hybridization arrays (CGHa) of bacterial artificial chromosomes (BACs) in the identification of tumor samples that carry loss of the 1p/19q chromosome arms. These BAC arrays carry approximately 6,000 BAC clones and provide an average inter-BAC resolution of 500 Kb. Using this approach we have clearly shown that 1p/19q loss in these cases, when compared with microsatellite-mediated detection of loss of heterozygosity, is due to physical hemizygous deletion of the whole chromosome arms in all cases. Furthermore, CGHa allows the simultaneous definition of the other genetic changes that are occurring in the tumors. From our survey of 14 tumors consisting of low-grade oligodendrogliomas (n = 6), anaplastic oligodendrogliomas (n = 5), or mixed oligoastrocytoma (n = 3), we were able to demonstrate the presence of additional genetic markers that were characteristic of the various grades of tumors as well as novel changes that had occurred. Thus, CGHa provides a robust, high throughput, genome-wide analysis of genetic changes of oligodendroglial tumors that can be used not only to predict chemo-responsiveness but also place these genetic changes in the context of other abnormalities in the same experiment without the need for extensive chromosome or LOH analysis.

Key Words: Bacterial artificial chromosome arrays; Chemo-responsiveness; Chromosome abnormalities; Comparative genome hybridization; Oligodendrogliomas.

INTRODUCTION

Diffuse gliomas are the most common primary tumors of the central nervous system affecting adults (1). Traditionally, the classification and grading system of glial tumors has been based on histological features, such as resemblance of astrocytic or oligodendrocytic cells and proliferation rate. The World Health Organization classification of gliomas recognizes 3 major histological types of tumors: astrocytomas, oligodendrogliomas, and oligoastrocytomas (2, 3) as well as ependymomas. Each type can be further divided into 2 or 3 malignancy grades. The terms, low grade (Grade II) and anaplastic (Grade III), are applied to all of these gliomas, whereas the highest grade, glioblastoma multiforme, is typically reserved for the most malignant neoplasms with an astrocytic phenotype. In general, prognosis has been shown to correlate roughly with classification and grade. The diagnosis of oligodendrogliomas, however, is frequently complicated by the fact that many tumors contain an astrocytic component. Furthermore, the pathology of these tumors is very complex and it is not unusual that even highly trained neuropathologists disagree with a particular diagnosis (4). The downstream consequences of this confusion are that, since histology may be the basis for entering a patient on a clinical trial, cross trial comparisons of outcome and response to treatment could be misleading. The correct diagnosis of tumors of oligodendroglial origin (including oligoastrocytomas) is particularly important because 60% to 70% of anaplastic oligodendrogliomas (AO) are sensitive to a combined chemotherapy and their prognosis is significantly better than that for astrocytic tumors of corresponding grades (5, 6). In contrast, despite a wealth of accumulated information, treatment of the advanced astrocytic tumors (anaplastic astrocytomas [AA] and glioblastomas [GBM]) is still largely unsuccessful due to their resistance to chemotherapy or radiotherapy. This difference in clinical behavior is thought to reflect the difference in genetic alterations and gene expression between astroglial and oligodendroglial tumors.

Cytogenetic and molecular studies of glial tumors performed during the last decade have identified several recurrent, nonrandom genetic abnormalities associated with glial tumors (7–10). Loss of heterozygosity (LOH) studies, in particular, have played a pivotal role in identifying the regions of the chromosomes that are repeatedly deleted in particular subgroups of the glial tumors, pinpointing the position of the genes whose inactivation is critical for tumorigenesis. The most frequent specific alteration
in oligodendroglial tumors, grades II to III, is the coincident loss of chromosomal arms 1p and 19q in 50% to 80% of the tumors (11–13). Remarkably, AOs with loss of 1p are also highly sensitive to PCV (procarbazine, lomustine, and vincristine) therapy, whereas AOs (which retain 1p) as well as AAs (which rarely show LOH for 1p) are highly resistant to chemotherapy. These observations have been used to predict whether AOs will be sensitive or resistant to chemotherapy (14, 15). This particular LOH assay provides a very important test for the clinical management of patients with AO. Clearly, however, it is essential to establish the LOH status of these tumors quickly and accurately in order to be able to customize therapy and begin it in a reasonable time following biopsy.

Currently, the main methods used to establish the LOH status of tumors involves using FISH analysis and microsatellite repeat marker analysis from DNA isolated either from fresh tissue or paraffin-embedded sections. Although highly successful, the LOH/FISH analysis must be carried out on a marker-by-marker basis and cannot place the specific 1p/19q loss in the context of other genetic changes without further extensive analysis. Recently, quantitative PCR has been developed using primers from CA-repeats, which can simultaneously analyze multiple loci on chromosome arms 1p and 19q (16). This approach speeds the analysis but still only surveys the chosen regions. More recently (17–20), high-resolution analysis of human tumors has been achieved using arrays of bacterial artificial chromosomes (BACs) in a comparative genomic hybridization approach (CGHa). The array currently available in our group consists of over 6,000 BACs spanning the length of the human genome at an average inter-BAC resolution of 500 Kb. In a single hybridization experiment, genetic deletions and amplifications in the tumor sample can be defined with high resolution (21). We have investigated the application of this approach to the analysis of brain tumors with oligodendroglial components and demonstrate that 1p/19q loss can be readily identified as well as the other genetic changes that are present in the tumor cells.

MATERIALS AND METHODS

Collection of Glial Tumors

All the brain tumors used in this study were collected in accordance with full IRB approval. Tumor tissues were obtained at the time of surgery and snap-frozen in liquid nitrogen. DNA samples were prepared from these tumors using standard phenol/chloroform procedures.

BAC Array Generation

A genome-wide resource of ~6,000 FISH-mapped, gene/marker content-verified, sequenced BAC clones (22) from the RPCI-11 human BAC library are represented as immobilized DNA targets on glass slides for array-based CGH analysis, as previously described (20). Each clone is spotted in triplicate at 280 μm intervals (see http://genomics.roswellpark.org for a complete list of clones).

DNA Preparation

Genomic DNA was prepared from all samples using FlexiGene DNA Isolation kit (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions. Two control DNA pools are used for BAC CGH array analysis. The male control and female control pools each contain DNA from 15 cytogenetically normal individuals. For procedural quality control, all analyses are performed as sex-mismatch hybridizations.

Labeling of DNA

One μg of control and test genomic DNA was labeled by random priming using a BioPrime DNA labeling kit (Invitrogen, Inc., Carlsbad, CA) for 3 hours at 37°C with the appropriate Cyanine dye (Cy3 or Cy5). After ethanol precipitation, the probes were resuspended in H2O and combined. Unincorporated Cy dye is removed by passage over a Qiagen spin column. The labeled probes are dried and stored at −20°C until hybridization.

Hybridization

Briefly, the arrays are preblocked with 110 μl Ambion SlideHyb Buffer #3, 1 μl of 20 μg/μl Human Cot-1 DNA solution at 50°C in a GeneTAC hybridization station (Genomic Solutions, Inc. Ann Arbor, MI) for 30 min. Prior to hybridization, the probe is resuspended in 110 μl Ambion SlideHyb Buffer #3 containing 5 μl of 20 μg/μl Cot-1 and 5 μl of 100 μg/μl yeast tRNA, heated to 95°C for 5 min and placed on ice. The prehybridization buffer is removed, the entire probe is added to the hybridization chamber, and hybridization proceeds for 16 hours at 65°C in the GeneTAC. After hybridization the slide is washed in decreasing concentrations of SSC and SDS to a final stringency of 0.1 × SSC at 65°C.

Image Analysis

The hybridized slides are scanned using an Affymetrix 428 Scanner to generate high-resolution (10 μm) images for both Cy3 and Cy5 channels. Image analysis is performed on the raw image files using ImaGene (V4.1BioDiscovery, Marina Del Rey, CA). Each spot is defined by a circular region, the size of which is programmatically adjusted to match the size of the spot. A buffer region of 2 to 3 pixels around the spot is ignored and a region 2 to 3 pixels wide outside of the buffer region is considered the local background for that spot. Each spot and its background region are segmented using a proprietary optimized segmentation algorithm, which excludes pixels that are not representative of the rest of the pixels in that region. The background-corrected signal for each BAC is the mean signal (of all the pixels in the region) minus the mean local background. The output of the image analysis is in the form of 2 tab-delimited files, one for each channel, containing all of the fluorescence data.

Data Analysis

The output of the image analysis is processed by a program written in Perl+R that was developed at Roswell Park Cancer.
Institute. For each spot, a ratio is calculated from the background subtracted mean signal of the 2 channels. The ratios are then normalized on the log scale with a nonlinear normalization algorithm. Basically, for all spots that are flagged as having met qualitative spot criterion, the log2 background subtracted mean signal is plotted and a lowess function is applied. The normalized ratios are the computed ratio minus the expected value on the curve.

The results of the triplicate replicates are combined by taking the mean of the log2 ratios and the standard error is calculated. Any BAC that has less than 2 replicates flagged as having met qualitative spot criterion is excluded. Mapping information is added to the resulting ratios and standard errors. The mapping data for each BAC is found by querying the human genome sequence at http://genome.ucsc.edu. The Nov. 14, 2002 build is currently being used to precisely position the BAC clones on the draft sequence. The output, a tab delimited file, is imported to Excel for graphing.

**Interpretation**

The final ratio represents relative amounts of DNA from the experimental sample and the reference control sample. Equal amounts of control and test DNA are labeled and the ratios are normalized to 1 (0 on the log scale) effectively normalizing the array to the average modal number of the test sample. Typically, a degree of suppression is observed in these ratios that is most likely due to the presence of repetitive DNA sequences in the BACs that compete equally for both probes. A more complete discussion of this issue was presented elsewhere (21). The X chromosome, however, can be used to estimate the amount of suppression when the test sample has a normal number of sex chromosomes (i.e. XX or XY). In general, background variation was considered to extend between ratios of 1.2 and 0.8 for diploid tumors on the linear scale and hybridization ratios outside of these values were considered losses or gains of genetic material.

**RESULTS**

To assess the usefulness of CGHa for the identification of LOH events in low-grade brain tumors, we used DNA from a series of 3 different tumor subtypes whose LOH status had previously been determined using microsatellite markers (23). The tumors had been grouped according to histopathological diagnosis and divided into low-grade oligodendrogliomas (LGO), anaplastic oligodendrogliomas (AO), and mixed oligoastrocytomas (MOA). From the original series of tumors classified using microsatellite markers, we analyzed representative samples that consisted of 6 LGOs, 5 AOs, and 3 MOAs. In each of these groups were examples of tumors where there was clear presence or absence of LOH for at least one of the microsatellite markers on each of the 1p and
19q chromosome arms. The CGHa profiles were compiled from approximately 6,000 discrete sites along the length of the human genome and an example of the full profiles that are obtained is shown in Figure 1.

In all cases in which the microsatellite analysis had suggested LOH, the CGHa identified loss of genetic material. Furthermore, in all cases, the LOH was clearly a result of physical loss of the whole of both of the 1p and 19q chromosome arms (Fig. 2). These data clearly demonstrate that CGHa can be used as a reliable and high throughput approach for predicting clinical outcome for patients with oligodendrogliomas. The advantage of CGHa is that it also produces a genome-wide survey of other genetic changes that are occurring on a background of 1p/19q loss. Structural chromosome changes identified using CGHa can be described in terms of their cytogenetic band nomenclature for ease of communication, but the relative location of the breakpoints can also be defined by the megabase position (Mbp) on the BAC along the length of the chromosome relative to the DNA genome sequence from the telomere of the short arm (defined as ‘0’). Within the LGOs, 2 tumors (#94 and #137) that had not shown LOH using microsatellite markers showed normal ploidy for 1p and 19q. In #137, however, there was an increase in genetic material on the distal long arm of chromosome 7 from Mbp 84.53-qter (Fig. 3). The other structural change involved the distal long arm of the X chromosome with a deletion from Mbp 81.61-qter (Fig. 3). Although it may appear that these are independent events, our experience with these types of CGHa changes (20) is that this probably represents an t(X;7) translocation where, as a result of the rearrangement, extra material on 7 is generated at the expense of the Xq material. The position of the breakpoint in this case can be defined to within 1 Mbp of the breakpoint and lies between the BACs where the abrupt change in signal ratios change. Within the CGHa karyotype of tumor #94 a homozygous deletion of a small region in 2q was the only detectable change (Fig. 3).

Since CGHa surveys the complete karyotype of the tumor, it is possible to describe the other changes that occur in tumors with loss of 1p and 19q. In 2 LGOs that showed LOH using microsatellite markers (#149 and #117), it was clear (Fig. 2) that it was the whole of the 1p and 19q arms that were deleted and that these were the only genetic changes we could detect at this level of resolution. In 2 additional cases (#10 and #113) the loss of 1p and 19q was accompanied by other genetic changes, suggesting a more unstable genome (Table).

Analysis of Anaplastic Oligodendrogliomas

AOs are considered a more advanced stage tumor. In our series of AOs (23), loss of 1p and 19q was also a common feature and again, in those tumors where LOH had been detected (#152, #100, #164) using microsatellite analysis, the whole of the 1p and 19q arms were lost, as seen in the LGOs. Tumor #100 revealed additional changes in the karyotype (Table) that included hemizygous loss of the region on 9p, which carries the CDKN2A (p16) gene. In contrast, tumor #164 showed 2 additional changes; an extra copy of chromosome 7 and loss of 1 copy of chromosome 15. Tumor #152 showed loss of 1 copy of chromosome 13 and an apparently homozygous deletion of a 3.6-Mbp region on the long arm of chromosome 18 (Fig. 4) as well as a hemizygous deletion of a 2.7-Mbp region on the long arm of chromosome 8. The AOs that did not show LOH for 1p and 19q (#78 and #91) showed some common...
Fig. 3. Examples of chromosome abnormalities identified in low-grade gliomas. The gain of chromosome 7 material and loss of X chromosome material in tumor #137 almost certainly indicate the presence of an unbalanced chromosome translocation with the breakpoints at the site indicated by the arrows. The deletion on 2q (arrow) in tumor #94 identifies a homozygous deletion. In the lower profile the subterminal deletion in the 1p36 region in tumor #78 spanning approximately 10 Mbp is shown (arrow). and some unique changes. Tumor #91 showed a deletion of most of the long arm of chromosome 4 and a DNA content increase in a region spanning Mbp 123.49 to 145.67 on distal 8q. In addition, a deletion of most of the short arm of chromosome 9, including the CDKN2A gene, and an extra copy of chromosome 12 was observed (Table). Chromosome 11 showed loss of material from the entire short arm and an increase of 2 regions of the long arm spanning Mbp 59.05 to 93.54 and Mbp 107 to 128.69, although the intervening region was underrepresented in the karyotype (Fig. 4). The mechanism behind these complex changes is difficult to establish based on CGHa, although the net result in term of loss of genetic material is clearly seen. Two other rearrangements in this tumor involved an interstitial deletion (42.49–72.22 Mbp) on the long arm of chromosome 15 (Fig. 4) and an extra copy of most of the long arm of chromosome 16. AO #78 also showed more extensive chromosome changes in the absence of total loss of 1p and 19q. In fact, this tumor showed a small deletion in the distal region of 1p involving 10.62 Mbp (Fig. 3) and a deletion of distal 4q (122.99-qter). An almost complete extra copy of chromosome 7 was seen, excluding the distal tip of the short arm between 0 and 6.15 Mbp, as well as loss of a whole copy of chromosome 10. Finally, loss of the proximal part of chromosome 13 from 0 to 86.8 Mbp was observed. In all, therefore, AOs show more genetic instability than was seen for the LGOs, which is typical of higher-grade tumors (Table).

Part of the classification of brain tumors describes mixed tumors that have both astroglial and oligodendroglial features (MOA). These are relatively rare and so only 3 samples were included in our series. In tumor #122, which also shows complete loss of 1p and 19q, there were apparently no other obvious changes making this similar to the CGHa profile seen for many of the LGOs. Tumor #74 showed only minimal changes with loss of a copy of chromosome 22, whereas tumor #151 showed more extensive changes with terminal deletions on the distal tips of 2q and 3q (Fig. 4), an extra copy of chromosome 7, and deletion of 9p. In this case, chromosome 10 was apparently normal. The only other change was a loss of a whole copy of chromosome 21. DISCUSSION

Cytogenetic changes in tumor cells have been used extensively to distinguish different subgroups within a tumor type but, in our experience, short-term culture of
low-grade tumors results in the rapid overgrowth by normal cells from the sample. Conventional CGH (24, 25) has been very successful in identifying genetic changes (26–28) but has low resolution and must be performed on a metaphase-by-metaphase basis. As a consequence, 1p/19 loss is routinely detected using FISH or standard LOH approaches using PCR and microsatellite markers, both of which are time consuming and labor-intensive approaches. Here we demonstrate that CGHa provides a rapid and unequivocal means of identifying 1p/19q loss. The CGHa analysis also addresses the mechanism of LOH in these tumors, where we clearly demonstrate that 1p/19q LOH events in low-grade brain tumors result from physical loss of genetic material that can be readily detected by CGHa analysis. CGHa has the advantage of a 24-hour turnaround time and requires only small amounts of DNA but most importantly, and in contrast to microsatellite and FISH analysis, can survey most of the other genetic changes that are present in the tumor cells at high resolution and at the same time in a single hybridization experiment. The advantage of this whole genome capability is further reinforced by the observation that loss of the short arm of chromosome 9, involving the CDKN2A locus, can be readily detected in the same analysis. This coincident observation has important implications for prognosis, since loss of this region is associated with poorer survival (14).

The primary aim of our study was not to characterize all of the genetic events that were seen in the CGHa profiles, but rather to assess the usefulness of CGHa in identifying 1p/19q loss in tumors with components of an oligodendrogial histology. In the data presented here, however, it is clear that this approach provides a rapid, objective, high throughput capability which, unlike LOH studies, can put the 1p/19q losses in the context of other numerical chromosome changes that have occurred in the tumor and at a far greater resolution than conventional CGH. These observations could be particularly relevant since, with more extensive analysis, the nature of these changes offers the possibility to further define subgroups of tumors that might have a different clinical response.

Losses of chromosome 10 and gains of all or parts of chromosome 7, for example, have frequently been reported as markers of more advanced stage glioma progression (29, 30). Extra copies of the long arm of chromosome 7 have also reportedly been associated with deletion of the 9p region containing the CDKN2A gene. Also associated with deletion of the 9p region containing the CDKN2A gene is poorer survival in astrocytomas (31). We were able to identify +7 or −10 in some of the AOs and MOAs analyzed with CGHa, which could potentially mark these tumors as more aggressive (32), especially when this is also associated with deletion of the 9p region containing the CDKN2A gene.

Within the CGHa profiles presented here, we have detected some interesting genetic changes that further support CGHa as a discovery tool. Identification of small genetic changes offers the opportunity to use positional cloning approaches to identify genes located in the regions that are related to tumorigenesis. For example,
LOH for restricted regions on the short arm of chromosome 1 has been detected in a subset of brain tumors (33) in which the combined data suggest a restricted region of common overlap in 1p36. In tumor #78, for example, we were able to define a small region of loss in 1p36 that may define the sublocation of a critical gene. In other tumors we were able to define small deletions that involve only a single BAC. In these cases, however, it will be important to verify that these changes are real using either FISH or PCR (20, 21). For example, the deletion on chromosome 18q in tumor #152 appears to be homozygous which, if verified, could mark the location of a tumor suppressor gene.

One of the hallmarks of GBM is the amplification of the EGFR gene in 7p12 (34, 35). In our previous study of GMB we were able to detect 10- to 30-fold amplification of EGFR in approximately 40% of these tumors (20). None of the low-grade tumors in this study, however, demonstrated amplification of EGFR, although several of the AOs and MOAs showed either extra copies of chromosome 7, or parts of chromosome 7. These observations are consistent, therefore, with the suggestion that EGFR amplification is associated with high-grade tumors.

In these studies we have been able to analyze DNA from fresh-frozen tissue. Since fresh tissue will not always be available we have investigated whether formalin-fixed paraffin-embedded tissue can be used for CGHa arrays (20, 21). In our preliminary studies it is clear that major genetic changes can be detected, although the quality of the CGHa karyotypes is clearly inferior to those derived from fresh tissue. We are currently assessing whether the 1p/19q loss can be reliably detected using archival material, which will allow this approach to be extended more generally to oligodendroglial tumors.

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Fig. 4. An example of a homozygous deletion on the long arm of chromosome 18 in tumor #152. A more complex rearrangement is seen for chromosome 11 in tumor #91 with loss of most of the short arm and gains of 2 specific regions on the long arm. An interstitial deletion on the short arm of chromosome 15 (between arrows) is also seen in tumor #91. Small deletions at the distal end of chromosomes 2 and 3 are seen in tumor #151.
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