0023-6837/01/8105-717$03.00/0
LABORATORY INVESTIGATION
Vol. 81, No. 5, p. 717, 2001
Printed in U.S.A.
Copyright © 2001 by The United States and Canadian Academy of Pathology, Inc.

Detection of Multiple Gene Amplifications in Glioblastoma Multiforme Using Array-Based Comparative Genomic Hybridization

Angela Bik-Yu Hui, Kwok-Wai Lo, Xiao-Lu Yin, Wai-Sang Poon, and Ho-Keung Ng

Department of Anatomical and Cellular Pathology (AB-YH, K-WL, X-LY, H-KN) and Neurosurgical Unit (W-SP), Department of Surgery, The Chinese University of Hong Kong, Hong Kong, China

SUMMARY: We have used a new method of genomic microarray to investigate amplification of oncogenes throughout the genome of glioblastoma multiforme (GBM). Array-based comparative genomic hybridization (array CGH) allows for simultaneous examination of 58 oncogenes/amplicons that are commonly amplified in various human cancers. Amplification of multiple oncogenes in human cancers can be rapidly determined in a single experiment. Tumor DNA and normal control DNA were labeled by nick translation with green- and red-tagged nucleotides, respectively. Instead of hybridizing to normal metaphase chromosomes in conventional comparative genomic hybridization (CGH), the probes of the mixed fluorescent labeled DNA were applied to genomic array templates comprised of P1, PAC, and BAC clones of 58 target oncogenes. The baseline for measuring deviations was established by performing a series of independent array CGH using test and reference DNA made from normal individuals. In the present study, we examined fourteen GBMs (seven cell lines and seven tumours) with CGH and array CGH to reveal the particular oncogenes associated with this cancer. High-level amplifications were identified on the oncogenes/amplicons CDK4, GLI, MYCN, MYC, MDM2, and PDGFRA. The highest frequencies of gains were detected on PIK3CA (64.3%), EGFR (57.1%), CSE1L (57.1%), NRAS (50%), MYCN (42.9%), FGR (35.7%), ESR (35.7%), PGY1 (35.7%), and D17S167 (35.7%). These genes are suggested to be involved in the GBM tumorigenesis. (*Lab Invest 2001, 81:717–723*).

Gene amplification is regarded to be a reflection of genetic instabilities in solid tumor cells (Schwab, 1999). Activation of proto-oncogenes by amplification is proposed to play an important role in the development of many human solid tumors. Detection of specific gene amplification in tumor cells can lead to the identification of cellular genes involved in growth control and tumorigenesis of cancers. Southern blotting, semi-quantitative or quantitative PCR, and fluorescence in situ hybridization (FISH) were commonly used to detect gene amplification in tumors. However, these techniques only examine one or a few genes in each experiment. Cytogenetic analysis can reveal amplification of multiple chromosomal regions at the same time. Nevertheless, amplifications detected in tumor cells by cytogenetic analysis may not be accurately interpreted because of the presence of complex chromosomal rearrangements. The technique of comparative genomic hybridization (CGH) has been widely used to screen for multiple chromosomal aberrations, including amplification and deletion, in various cancers (Kallioniemi et al, 1992). CGH is a valuable technique for analysis of solid tumors from which good quality metaphases are extremely difficult to obtain. However, it has a limited mapping resolution and genetic alterations at regions $<$ 20Mb may not be detected (Du Manoir et al, 1995). Small regions of genetic alterations usually are overlooked.

As a complement to CGH, we have implemented a new DNA microarray technology to investigate oncogene amplifications throughout the genome of glioblastoma multiforme (GBM). Array-based comparative genomic hybridization (array CGH) has been suggested as a new method for identification of oncogenes and provides precise locations of amplicon boundaries (Albertson et al, 2000; Pinkel et al; 1998). In the current study, CGH microarray templates containing 58 target clone DNA (P1, PAC, or BAC clones) were used (Genosensor CGH, Vysis, Downers Grove, Illinois). These clones have been reported to be associated with tumor formation through amplification. Similar to conventional CGH, tumor DNA and normal control DNA were labeled by nick translation with green- and red-tagged nucleotides, respectively. Instead of hybridizing to normal metaphase chromosomes in conventional CGH, probes of mixed fluorescence labeled DNA were applied to microarray templates. Copy-number changes of each of the 58 clones were investigated simultaneously at one assay. Hence, array CGH is useful for identification of multiple gene amplifications and evaluation of potential associations among them. In this study, fourteen GBMs (seven cell lines and seven tumors) were examined. CGH was applied to detect amplified chromosomal regions that may be harboring GBM-associated

Received January 18, 2001.
This research was supported by a grant from the Research Grant Council.
Address reprint requests to: Professor H. K. Ng, Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong, China. E-mail: hkng@cuhk.edu.hk

oncogenes. Afterward, the samples were studied further with array CGH to reveal the target oncogenes involved.

Results

Comparative Genomic Hybridization

Chromosomal imbalances were identified on all of the fourteen GBM cell lines and tumors, as shown in Figure 1a. The samples illustrated multiple aberrations, including both gains and losses of chromosomal materials. Consistent with previous reported CGH analysis of GBM, the most common chromosomal aberrations detected were gain of chromosome 7 and loss of chromosomes 9p and 10q (Bigner and Schrock, 1997; Mohapatra et al, 1998; Weber et al, 1996). Twelve of the fourteen cases (85.7%) demonstrated chromosome 7 gain. Ten cases had gain of whole chromosome 7, whereas the other two cases had 7q gain only. Loss of chromosomes 9p and 10q

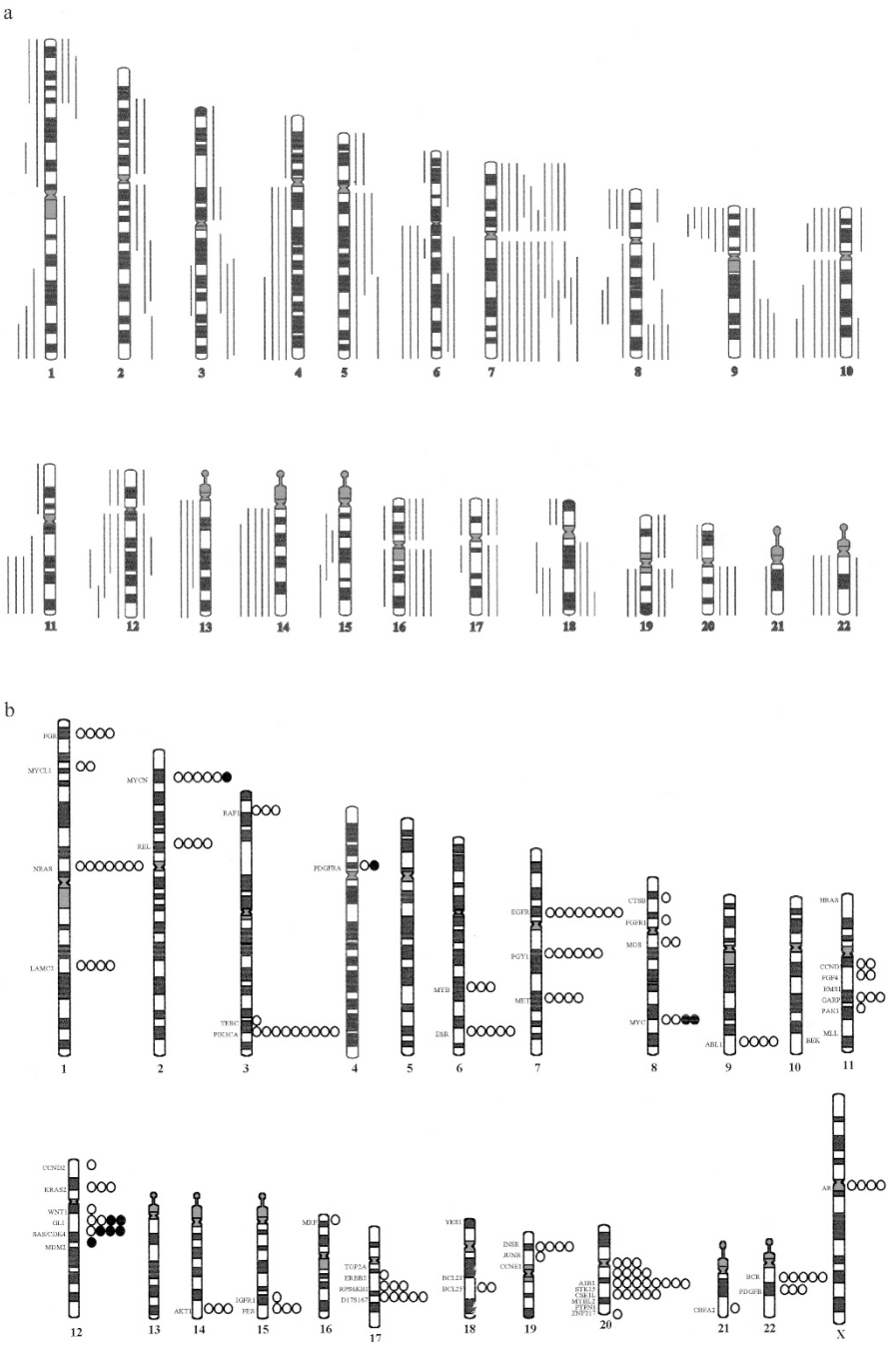

Figure 1.

Illustration of chromosomal imbalances detected in glioblastoma multiforme (GBM) cell lines and primary GBM samples by comparative genomic hybridization (CGH) analysis (a). Lines on the left and right side of ideogram indicating chromosomal gains and losses, respectively. Results of array CGH analysis of the 14 GBM tumors (b). Open circles represent gain of oncogenes, filled circles indicate gene amplifications.

was both detected in 42.9% (6 of 14) of the cases. Other frequently detected aberrations included loss of chromosome 4q (28.6%), 11q (28.6%), and 14q (35.7%) and gain of 9q (28.6%) and 16q (28.6%).

Array-Based CGH

Defining Limit for Measuring Deviations of Array CGH. To determine the variations of the green to red ratios on the oncogenes in normal control DNA, we studied five independent comparative hybridizations using test and reference DNA made from normal individuals. Fluorescence ratios on the targets were measured to establish the baseline for measuring deviations. The measured ratios ranged from 0.81 to 1.19. Mean ratio and standard deviation of the normal array CGH were 1.0 and 0.09, respectively. A value of the mean ratio plus two standard deviations (mean $\pm$ 2 SD for all 5×58 targets $= 290$ measurements) was set as the cut-off level for the normal gene copy number. Hence, ratios > 1.18 and < 0.82 were regarded to have deviations. Green to red ratios $>$ 2.18 ($> 2 + 2$ SD) were regarded to have amplifications. Reproducibility of our results was further confirmed with two self-to-self comparisons. Genomic DNA was differentially labeled with green and red fluorescence and hybridized to microarray templates. The resulting green to red ratios of the target clones were around 1 and ranged from 0.8 to 1.18. Hybridization of normal female and normal male individuals was conducted to compare the one extra copy of X chromosome from the normal female DNA. The measured fluorescence ratio of the extra copy of AR gene (located at Xq11-q12) ranged from 1.49 to 1.52.

Array CGH Analysis of GBM Cell Lines and Tumors. After CGH analysis, DNA of the GBM cell lines and tumors was analyzed with array CGH. Fluorescent ratios of the green-to-red-labeled DNA ranged from 0.57 to 5.26. According to normal controls, ratios $>$ 1.18 (mean + 2 SD) were scored to have gain of a particular oncogene, and ratios < 0.82 (mean $-$ 2 SD) were counted to have losses. Results of array CGH analysis are illustrated in Figure 1b. With this criteria, gain of genes was detected on most of the genes except BEK, HRAS, EMS1, MLL, WNT1, IGFR1, TOP2A, YES1, BCL23', CCNE1, and PTPN1. The GBM cell lines and tumors showed similar frequency of gain or amplification of these oncogenes. Frequency of gains ranged from 0 to 64.3%. The mean percentage and standard deviation of gain was 18.8% $\pm$ 15.6%. In the current study, 34.4% (mean percentage + 1 SD: 18.8% $\pm$ 15.6%) was chosen to be a cut-off baseline representing significant percentage of gain of oncogenes. This represents the 95% confidence upper limit for the overall rate of random gain of genes. Gain of PIK3CA (64.3%), EGFR (57.1%), CSE1L (57.1%), NRAS (50%), MYCN (42.9%), FGR (35.7%), ESR (35.7%), PGY1 (35.7%), and D17S167 (35.7%) were higher than this baseline level. Consistent loss of AKT1 gene was detected in six of the fourteen cases (42.9%). Moreover, gene amplification was observed on CDK4 (21.4%), GLI (14.3%), MYCN (7.1%), PDGFRA (7.1%), MYC (7.1%), and MDM2 (7.1%). Cases with gain of multiple candidate oncogenes were analyzed further to determine the presence of co-amplified genes. Because only a limited number of tumor samples were examined in this study, no significant association was demonstrated among the oncogenes that illustrated gains.

Discussion

We have used the genomic microarray for simultaneous investigation of amplification of 58 human oncogenes/amplicons throughout the genome of GBM. This array-based CGH can analyze multiple oncogenes commonly amplified in various human cancers. In array CGH, the probe of the tumor DNA and normal control was hybridized to a CGH microarray template containing P1, PAC, and BAC clones of the 58 target oncogenes. This allows rapid investigation of the association and correlation of the abnormalities in multiple oncogenes. Analysis of gene amplification has been studied extensively in GBM. Oncogenes reported to be associated with the development of GBM include EGFR, CDK4, MDM2, GLI, PDGFRA, MET, MYC, etc (Collins, 1993; Galanis et al, 1998; Muleris et al, 1994; Olson et al, 1998; Reifenberger et al, 1996). Among these previous studies, only a few candidate oncogenes were investigated at a time. Besides EGFR, the most commonly amplified oncogenes including CDK4/SAS and MDM2, which are located in the 12q13-q15 region (Galanis et al, 1998; Olson et al, 1998; Reifenberger et al, 1996). In concordance with previous GBM studies, array CGH demonstrated high levels of gene amplification on CDK4, GLI, MYCN, PDGFRA, MYC, and MDM2. Chromosomal gains, which were not previously reported, were detected on PIK3CA (64.3%), CSE1L (57.1%), and NRAS (50%).

In the current CGH analysis, 71.4% of the studied cases had frequent gain of chromosome 7p (10 of 14 cases). EGFR gene locates at 7p12.3–12.1. The result is concordant with that of the array CGH, which detected gain of EGFR in 57.1% of the cases. Loss of chromosome 14q was found in 35.7% of the cases by CGH, and array CGH detected loss of AKT1 gene (located in 14q13) in 42.9% of the cases. These indicate that CGH and array CGH illustrate consistent results in our studies.

CGH analysis can detect amplified chromosomal regions that may be harboring cancer-associated oncogenes. However, it has a limited mapping resolution in which genetic alterations at regions $<$ 20Mb may not be detected. Small regions of genetic alterations are usually overlooked or uncounted. Regions with complex aberrations that contain both gain and loss of genetic materials may not be properly elucidated. As a complement to CGH, microarray-based CGH is effective in targeting the particular oncogenes of the new amplified regions revealed by CGH. In Figure 1b, 64.3% of the samples were found to have gains in the gene PIK3CA (located at 3q26.3). However, gain of the nearby oncogene TERC was detected in only one of

the cases. Gain of chromosome 3q (containing the PIK3CA gene) was found in 14.3% of the cases by this CGH analysis. This is similar to a previously reported CGH analysis of GBM that identified 3q26–28 to be one of the amplification sites (Mohapatra et al, 1998). Our findings suggest that alteration of PIK3CA may be involved in the GBM tumorigenesis. PIK3CA encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3-kinase). PI3-kinase mediated signaling was associated with cell proliferation, glucose transport and catabolism, cell adhesion, apoptosis, RAS signaling, and oncogenic transformation (Jimenez et al, 1998). In addition, AKT1 and AKT2 are downstream effectors of PI3-kinase, which has been reported to be activated through this gene (Jimenez et al, 1998; Shayesteh et al, 1999). To confirm the gain of PIK3CA, the GBM cell lines were examined with FISH analysis using the BAC DNA of this gene. Centromere probe of chromosome 17 was used as a control. For cell line GBM 6840, the green to red ratio detected on PIK3CA gene was 1.33, indicating the presence of an extra copy of that gene. Most of the cells and metaphases of GBM 6840 illustrated three green signals, showing that the cell line is triploid. FISH analysis of this triploid cell line illustrated the presence of four red signals in approximately 70% of the cells and three signals in 24% of the cells (Fig. 2). This confirmed the presence of an extra copy of PIK3CA gene in this cell line.

CGH detects amplifications in relatively large chromosomal regions. It is unable to precisely target the oncogenes involved. As in our tumor GBM#1, high levels of amplification were found around chromosome 12q13-q21 by CGH analysis (Fig. 3a). Several candidate oncogenes, such as WNT1, GLI, CDK4/SAS, MDM2, etc., were located on 12q13. With array CGH (Fig. 3b), the oncogenes amplified in this sample were clearly shown to be SAS/CDK4 (3.28) and MDM2 (4.9) only, but not WNT1 (0.91) or GLI (1.05). Thus, we are able to exclude the involvement of the WNT1 and GLI genes in this tumor.

Array CGH is a useful tool for the identification of molecular changes in understanding the genetics of cancer and identifying potential genetic markers to correlate with patient outcome and design adjunct therapy. It allows for rapid detection of copy number changes of target oncogenes located in small regions of the genome and multiple amplified oncogenes that are involved in the tumorigenesis. With development of a higher resolution array that encompasses more clones of candidate oncogenes and tumor suppressor genes, it may reveal even more complex events related to the development of cancers.

Materials and Methods

Patients

Seven cases of primary GBM were retrieved from the Brain Tumor Bank in the Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong. All of the cases were histologically diagnosed to be primary GBM according to the World Health Organization classification. The primary tumor tissues were snap-frozen and stored at −70° C until DNA extraction.

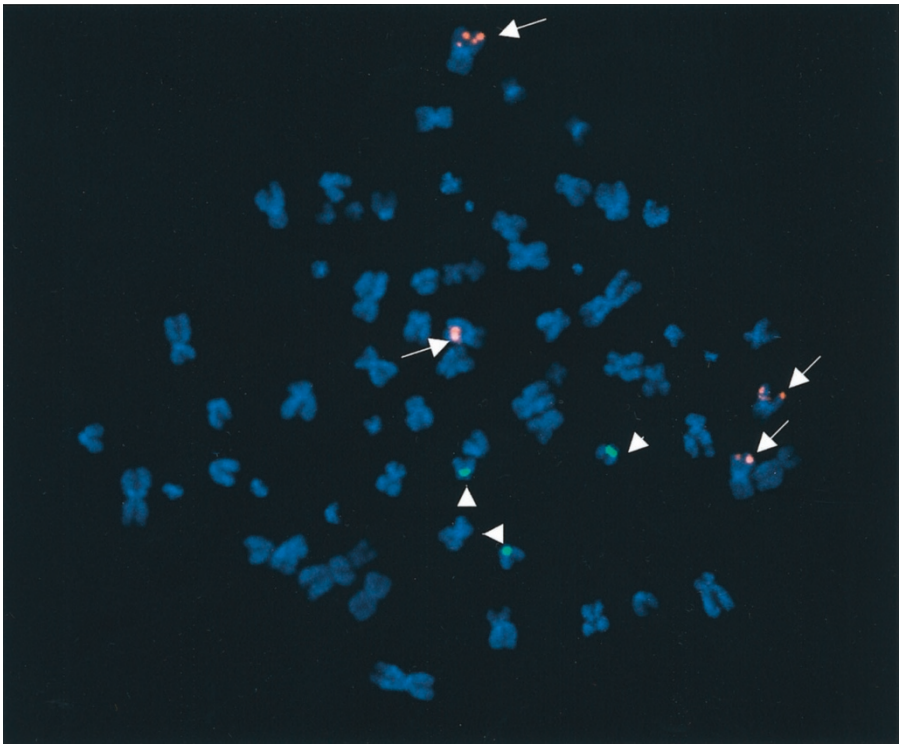

Figure 2.

Result of fluorescence in situ hybridization analysis in a GBM cell line (GBM 6840) with gain of PIK3CA (green-to-red ratio: 1.33) by array based CGH. Green signals (×3) refer to centromere probe of chromosome 17 to indicate the triploid status of the cell line. Red signals (×4) represent gain of one extra copy of PIK3CA gene.

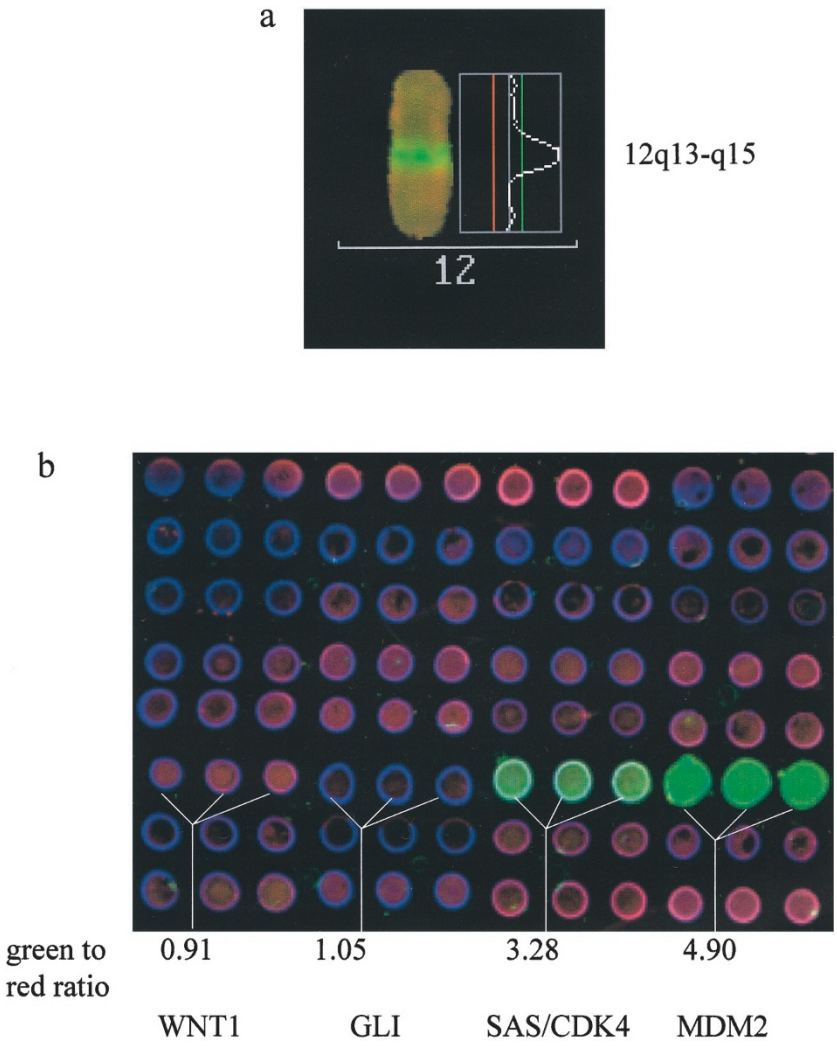

Figure 3.
Ratio profile demonstrating gain of chromosome 12 in a GBM tumor (GBM#1) (a). The high intensity fluorescent band indicates the amplification of 12q13–21 region. Illustration of array CGH study showing high-level amplification of SAS/CDK4 and MDM2 amplification in tumor GBM#1 (b).

Cell Lines

Cell lines A172 (CRL 1620, American Type Culture Collection, Rockville, Maryland), U373 MG (HTB 17, American Type Culture Collection), U343, D24.7, LNZ 308 were cultured with Dulbecco's Modified Eagle's Medium (GIBCO BRL, Gaithersburg, Maryland) supplemented with 10% fetal bovine serum (GIBCO BRL) and 1% penicillin/streptomycin (GIBCO BRL). Two other cell lines, GBM 6840 and GBM 2603, were established from the tumor tissues of two GMB patients in our laboratories. These two cell lines were cultured with the same medium.

DNA Extraction

High-molecular-weight DNA of both the primary tumors and cell lines were isolated as described in Maniatis et al (1989). The cell pellets or primary tissues were digested overnight in saline tris-EDTA (STE) solution containing 0.5% sodium dodecyl sulfate and 0.2 mg/mL proteinase K (Sigma, St. Louis, Missouri) at 55° C with shaking. After overnight incubation, phenol/chloroform extraction was performed to denature and remove the protein content. In brief, the solution was mixed with phenol/chloroform/isoamyl alcohol (25:24:1 in volume) solution (Amresco, Solon, Ohio) for 10 minutes and centrifuged at 4000 rpm for 20 minutes. The upper aqueous layer was collected, precipitated with 110 volume 3M sodium acetate (pH 5.2) and 2.2 volumes cold absolute ethanol, and then kept at −20° C for at least one hour. After washing with cold 70% ethanol, the pelleted DNA was air-dried and dissolved in tris-EDTA (TE) solution (pH 7.5). The remaining RNA in the DNA solution was digested with 0.1 mg/mL DNase-free RNase A (Sigma) at 37° C for 2 hours and then with proteinase K treatment at 55° C for one hour. The solution was further extracted with phenol-chloroform solution as described above.

Comparative Genomic Hybridization

The CGH protocols were performed as described previously (Hui et al, 1999). The primary GBM DNA

and GBM cell line DNA were labeled with biotin-16-dUTP (ROCHE, Germany) by nick translation. Control DNA from normal individuals were labeled with digoxigenin (dig)-11-dUTP (ROCHE, Mannheim, Germany). Eight hundred nanograms of biotinylated tumor or cell line DNA were coprecipitated with 800 ng of sex mismatched dig-labeled normal reference DNA and 50 ng of Cot-1 DNA (BRL Life Sciences, Gaithersburg, MD). The hybridization was carried for 3 days in a 37° C incubator. Biotinylated DNA was detected through avidin conjugated fluorescein isothiocyanate (FITC, Vector Laboratories, Burlingame, CA). Dig-labeled DNA was visualized with anti-dig-rhodamine (ROCHE). Chromosomes were counterstained with antifade solution containing 4,6-diamino-2-phenylindole (DAPI, Vector Laboratories).

Digital images (Rhodamine, FITC and DAPI) of hybridized metaphases were captured independently through three separate band pass filters by a cooled charge-coupled device (CCD) camera connected onto a Zeiss fluorescence microscope (Jena, Germany). In each case, at least 15 metaphases were acquired and analyzed. The averaged ratio profiles were calculated with a digital imaging system ISIS3 (Metasystems GmbH, Sandhausen, Germany). Threshold levels of chromosomal gains and losses were set at 1.25 and 0.75, respectively.

Array-Based CGH (Array CGH)

DNA labeling was carried with nick translation according to the manufacturer's protocol. Tumor and cell line DNA were labeled by nick translation with Alexa-488 (green, Molecular Probes), and the normal reference DNA was labeled with Alexa-594 (red, Molecular Probes). The resulting DNA was checked with 1% agarose gel to ensure that the size was $<$ 300bp. 500 ng–700 ng each of tumor DNA and reference DNA were mixed with microarray hybridization buffer that contained a high concentration of Cot-1 DNA (Vysis). The probe mixture was denatured at 80° C for 10 minutes and incubated at 37° C for 1–2 hours before being transferred to the AmpliOnc I microarray (Vysis). The microarray contains a target clone of 58 oncogenes/amplicons commonly amplified in human cancer. A list of the amplicons are shown in Figure 1b, and detail information can be obtained from www.vysis-.com. The sources of DNA were from P1, PAC, or BAC clones. Each of the 58 oncogenes was represented by three target spots. DNA clones of the desired target sequences were arrayed in target spots of approximately 100–250 μm diameter. After an overnight hybridization at 37° C, the microarrays were washed three times with 50% formamide/2XSSC at 40° C for 10 minutes each. Thereafter, the microarrays were subjected to four washes with 1XSSC at room temperature for 5 minutes each. The microarrays were left to air-dry in the dark. Target spots were counterstained with a blue fluorophore included in the DAPI IV mounting solution (Vysis).

Images of the green, red, and blue fluorophore were analyzed and captured with the GenoSensor Reader System (Vysis). The system contains a large-field multicolor fluorescence imaging system that captured an image of the hybridized chip in three color planes: green, red, and blue. Target spots were automatically identified by the software. The most representative ratio of the modal DNA copy number of the sample DNA was calculated by analyzing the set of green to red ratios on all of the targets. For each target, the normalized ratio relative to the modal DNA copy number was calculated. This normalized ratio of target indicated the degree of gain or loss of copy number when compared with the sample's modal copy number.

Acknowledgement

We thank Dr. K. F. To, Department of Anatomical and Cellular Pathology, the Chinese University of Hong Kong, China, for performing the statistic analysis.

References

Albertson DG, Ylstra B, Segraves R, Collins C, Dairkee SH, Kowbel D, Kuo WL, Gray JW, and Pinkel D (2000). Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 25:144–146.

Bigner SH and Schrock E (1997). Molecular cytogenetics of brain tumors. J Neuropathol Exp Neurol 56:1173–1181.

Collins VP (1993). Amplified genes in human gliomas. Semin Cancer Biol 41:27–32.

Du Manoir S, Schrock E, Bentz MR, Speicher M, Joos S, Ried T, Lichter P, and Cremer T (1995). Quantitative analysis of comparative genomic hybridization. Cytometry 19:27–41.

Galanis E, Buckner J, Kimmel D, Jenkins R, Alderete B, O'Fallon J, Wang CH, Scheithauer BW, and James CD (1998). Gene amplification as a prognostic factor in primary and secondary high-grade malignant gliomas. Int J Oncol 13:717–724.

Hui AB, Pang JC, and Ng HK (1999). Detection of chromosomal imbalances in growth hormone-secreting pituitary tumors by comparative genomic hybridization. Hum Pathol 30:1019–1023.

Jimenez C, Jones DR, Rodriguez-Viciana P, Gonzalez-Garcia A, Leonardo E, Wennstrom S, von Kobbe C, Toran JL, R-Borlado L, Calvo V, Copin SG, Albar JP, Gaspar ML, Diez E, Marcos MA, Downward J, Martinez-A C, Merida I, and Carrera AC (1998). Identification and characterization of a new oncogene derived from the regulatory subunit of phophoinositide 3-kinase. EMBO J 17:743–753.

Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, and Pinkel D (1992). Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818–821.

Maniatis T, Fritsch E, and Sambrook J (1989). Molecular cloning. In: Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 6.34–6.35.

Mohapatra G, Bollen AW, Kim DH, Lamborn K, Moore DH, Prados MD, and Feuerstein BG (1998). Genetic analysis of

glioblastoma multiforme provides evidence for subgroups within the grade. Genes Chromosomes Cancer 21:195–206.

Muleris M, Almeida A, Dutrillaux AM, Pruchon E, Vega F, Delattre JY, Poisson M, Malfoy B, and Dutrillaux B (1994). Oncogene amplification in human gliomas: A molecular cytogenetic analysis. Oncogene 9:2717–2722.

Olson JJ, Barnett D, Yang J, Assietti R, Cotsonis G, and James CD (1998). Gene amplification as a prognostic factor in primary brain tumors. Clin Cancer Res 4:215–222.

Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, and Albertson DG (1998). High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarray. Nat Genet 20:207–211.

Reifenberger G, Ichimura K, Reifenberger J, Elkahloun AG, Meltzer PS, and Collins VP (1996). Refined mapping of 12q13–15 amplicons in human malignant gliomas suggest CDK4/SAS and MDM2 as independent amplification targets. Cancer Res 56:5141–5145.

Schwab M (1999). Oncogene amplification in solid tumors. Semin Cancer Biol 9:319–325.

Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, and Gray JW (1999). PIK3CA implicated as an oncogene in ovarian cancer. Nat Genet 21:99–102.

Weber RG, Sommer C, Albert FK, Kiessling M, and Cremer T (1996). Clinically distinct subgroups of glioblastoma multiforme studied by comparative genomic hybridization. Lab Invest 74:108–119.