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Comparison of Non-amplified and Amplified DNA Preparation Methods for Array-comparative Gnomic Hybridization Analysis

Hong-Jin Joo[1,*], Seung-Hyun Jung[1,2,*], Seon-Hee Yim[2], Tae-Min Kim[1], Hai-Dong Xu[1,2], Seung-Hun Shin[1,2], Mi-Young Kim[1,2], Hyun-Mi Kang[1] & Yeun-Jun Chung[1,2]

[1]Department of Microbiology, The Catholic University of Korea, Seoul 137-701, Korea
[2]Integrated Research Center for Genome Polymorphism, The Catholic University of Korea, Seoul 137-701, Korea
Correspondence and requests for materials should be addressed to Y. J. Chung (yejun@catholic.ac.kr)
*Hong-Jin Joo and Seung-Hyun Jung contributed equally to this work.

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Abstract

Tumor tissue is usually contaminated by normal tissue components, which reduces the sensitivity of analysis for exploring genetic alterations. Although microdissection has been adopted to minimize the contamination of tumor DNA with normal cell components, there is a concern over the amount of microdissected DNA not enough to be applied to array -CGH reaction. To amplify the extracted DNA, several whole genome amplification (WGA) methods have been developed, but objective comparison of the array-CGH outputs using different types of WGA methods is still scarce. In this study, we compared the performance of non-amplified microdissected DNA and DNA amplified in 2 WGA methods such as degenerative oligonucleotide primed (DOP)-PCR, and multiple strand displacement amplification (MDA) using Phi 29 DNA polymerase. Genomic DNA was also used to make a comparison. We applied those 4 DNAs to whole genome BAC array to compare the false positive detection rate (FPDR) and sensitivity in detecting copy number alterations under the same hybridization condition. As a result microdissected DNA method showed the lowest FPDR and the highest sensitivity. Among WGA methods, DOP-PCR amplified DNA showed better sensitivity but similar FPDR to MDA-amplified method. These results demonstrate the advantage and applicability of microdissection for array-CGH analysis, and provide useful information for choosing amplification methods to study copy number alterations, especially based on precancerous and microscopically invaded lesions.

Keywords: Array-CGH (Comparative Genomic Hybridization), Microdissection, DOP-PCR (Degenerated Oligonucleotide-Primed PCR), Phi 29 DNA polymerase

Microarray-based comparative genomic hybridization (array-CGH) enables higher-resolution copy number analysis across the whole chromosome by single hybridization[1,2]. The resolution of array-CGH has been rapidly improved and tiling arrays are available now. This enormous technical advance has contributed to delineating clinically significant, minimally altered regions (MAR) especially in various cancers, which potentially include novel cancer-related genes[1,3,4]. Array-CGH is a powerful tool also for toxicogenomics studies, such as studying the lesions induced by carcinogens[5].

Precise identification of the genetic alterations in premalignant as well as microscopically invaded lesions has been one of the key issues of cancer research. Tumor tissue is invariably contaminated by stromal cell or normal connective tissue components, which reduces the sensitivity of array-CGH analysis for exploring genetic alterations when using genomic DNA extracted from a tumor tissue[6]. It means copy number changes, especially low-level changes, might not be readily detectable if surrounding normal cells are not removed. Johnson *et al* suggested that at least 70% of test material should contain tumor DNA for reliable array-CGH analysis[7].

Microdissection has been adopted as a powerful tool to minimize the contamination of tumor DNA with normal cell components, which increases sensitivity and specificity[6-8]. However, the amount of DNA extracted from microdissected tissue is often not enough to be applied to array-CGH reaction. To amplify the amount of extracted DNA, several whole genome

amplification (WGA) methods have been developed; multiple strand displacement amplification (MDA) using Phi 29 DNA polymerase, universal-primer based PCR amplifications such as degenerative oligonucleotide primed (DOP)-PCR and ligation mediated PCR[9,10]. MDA is known to be affected by the integrity of genomic DNA, while DOP-PCR is relatively less affected. Instead, there is a possibility of nonspecific or uneven amplifications in the DOP-PCR method, which can potentially deteriorate original complexity of test genome[11].

Although microdissected DNA becomes more and more commonly used for array-CGH, objective comparison of the array-CGH outputs using different types of DNA preparation approach is still scarce. In this study, we compared array CGH results which were obtained using DNA from the same patient, but prepared in different ways; 1) genomic DNA, 2) microdissected, non-amplified DNA, 3) WGA by MDA, 4) WGA by DOP-PCR.

Preparation of Four Different Types of DNA Samples from the Same Tissue

To examine the influence of the DNA preparation methods on array output, we applied 3K whole-genome BAC array on DNA originated from the same lung cancer tissue, but prepared in 4 different ways, under the same hybridization condition.

Through microdissection, approximately 200 ng of genomic DNA was harvested from ~3 × 3 mm area. We amplified part of the microdissected DNA by two commonly used WGA methods; MDA using Phi29 DNA polymerase and DOP-PCR. For DOP-PCR amplification, we used 3 kinds of DOP primers (DOP-1, 2 and 3) and merged the amplified products for array-CGH. Figure 1 illustrates the genomic amplification products by MDA- and DOP-amplification. Through amplification, the original amount of DNA increased 160-5,000 folds depending on the quality of template DNA and amplification methods. We will call the microdissected, not amplified DNA 'microdissected DNA' and total genomic DNA without microdissection 'genomic DNA'. For amplified DNA, DOP-PCR amplified microdissected DNA will be called 'DOP-PCR amplified DNA' and Phi29 DNA polymerase amplified microdissected DNA 'MDA-amplified DNA' throughout this paper.

General Description of Copy Number Alterations by Four Different Methods

We performed whole-genome array-CGH analysis using the same amount of DNAs (800 ng/hybridization) prepared by four different approaches under the same hybridization and washing conditions as described in the methods section. Cutoff value was set to be over ± 0.2 in $\log_2$ ratio for copy number changes (gain and loss, respectively) and over ± 1 in $\log_2$ ratio for high-level amplification and deletion. As shown in Figure 2, this lung cancer sample showed substantial amount of copy number alterations across the diverse chromosomes. Upper two plots (Figure 2 A and B) illustrate the results using unamplified DNAs (genomic DNA and microdissected DNA) and lower two plots (Figure 2C and D) the results using MDA and DOP-PCR amplified DNA. All four profiles are largely consistent; copy number gains on chromosomes 1q, 2p, 3q, 7q, 17q and 19; copy number losses on chromosomes 1p, 3p, 4, 6q, 7p, 14p, 18 and Y; 1.05 Mb-sized amplification on chromosome 7 (55.02 to 56.07 Mb). However, there are noteworthy differences in copy number profiles according to DNA preparation methods.

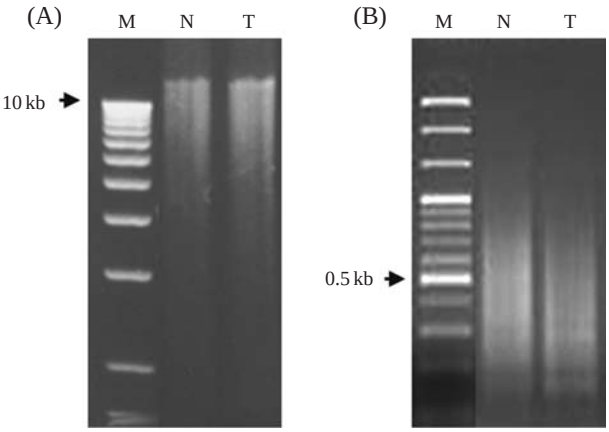

Figure 1. Agarose gel electrophoresis features of whole genome amplification products using microdissected DNA (A) MDA generated the amplification products mainly above 10 Kb size (B) DOP-PCR generated amplification products of which modal product size was approximately 400-500 bp. M, size maker (1 Kb ladder marker for A and 100 ladder marker for B); N, Normal; T, Tumor.

False Positive Detection Rates (FPDR) by DNA Preparation Methods

We firstly estimated FPDRs of array-CGH results by DNA preparation methods using the profile of genomic DNA as reference. To calculate FPDRs, we divided the number of BAC clones exceeding the cutoff value by total number of BAC clones in the chromosomes, which seem to be almost diploid in genomic DNA (i.e., chromosome 5, 10, 13, and 23). As a whole, FPDRs in both MDA and DOP-PCR amplified DNA were higher than that in genomic and microdissected DNA. Average FPDRs based on the chromosomes above were 7.5% (40/532), 37% (197/532), 4.5

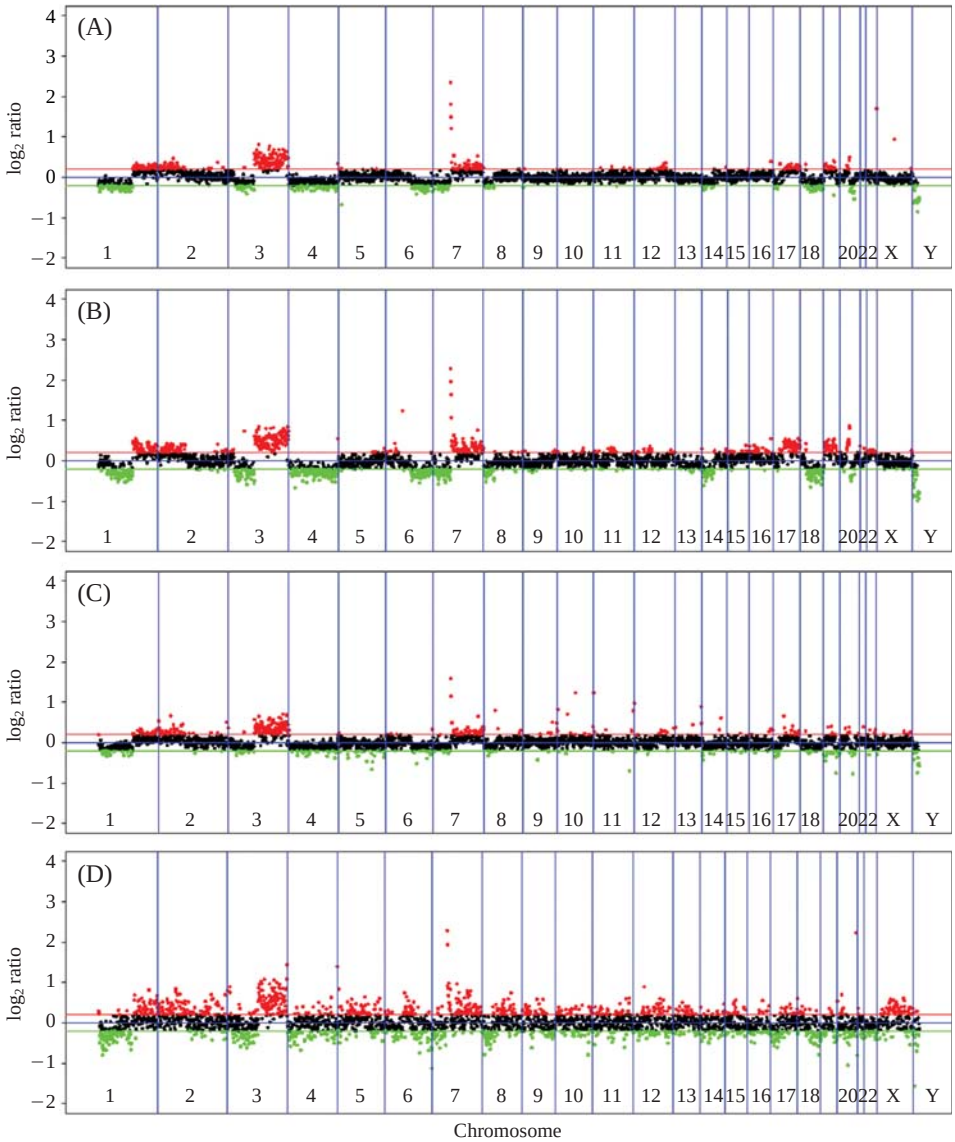

Figure 2. Whole genome array-CGH profiles of DNAs prepared in 4 different methods. (A) non-amplified genomic DNA (B) non-amplified, microdissected DNA (C) Phi 29 amplified DNA (D) DOP-PCR amplified DNA. X axis represents each chromosome and Y axis represents the signal intensity ratio (test/reference) in $\log_2$ ratio. Red and green lines represent the cutoff for copy number changes. Red dot, copy number gain; Green dot, copy number loss; Black dot; no copy number change.

% (24/532), and 1.5% (8/532), for MDA-amplified DNA, DOP-PCR amplified DNA, microdissected DNA, and genomic DNA, respectively. Figure 3A illustrates copy number profiles of chromosome 5 as an example of comparison. In MDA-amplified DNA, 17 of 170 clones (10%) showed false copy number changes and 61 (35.9%) in DOP-PCR amplified DNA. But, only 6 (3.5%) clones showed false copy number changes in non-amplified microdissected DNA.

Comparison of Sensitivity by DNA Preparation Methods

We also compared sensitivities of array-CGH results by DNA preparation methods. For doing this, we selected four typical copy number alterations in microdissected DNA, because copy number alterations were most apparent in microdissected DNA as we expected (Figure 3). In chromosome 18q, 53.44 Mb-sized (21.43-74.87 Mb) copy number loss was clearly identified from microdissected DNA, 73% of whose clones (44/60) in the region were above the cutoff level (Figure 3B). Although this copy number alteration was also detected in genomic DNA and DOP-PCR amplified DNA, the level of signal intensity ratio was around borderline in genomic DNA and it is hard to define the exact boundary of the alteration in DOP-PCR amplified DNA. In MDA-amplified DNA, this alteration was not detected.

In chromosome 20, 8.35 Mb-sized (26.00-34.35 Mb) copy number gain was identified in microdissected DNA, but the level of copy number gain was much lower in genomic DNA and almost undetectab-

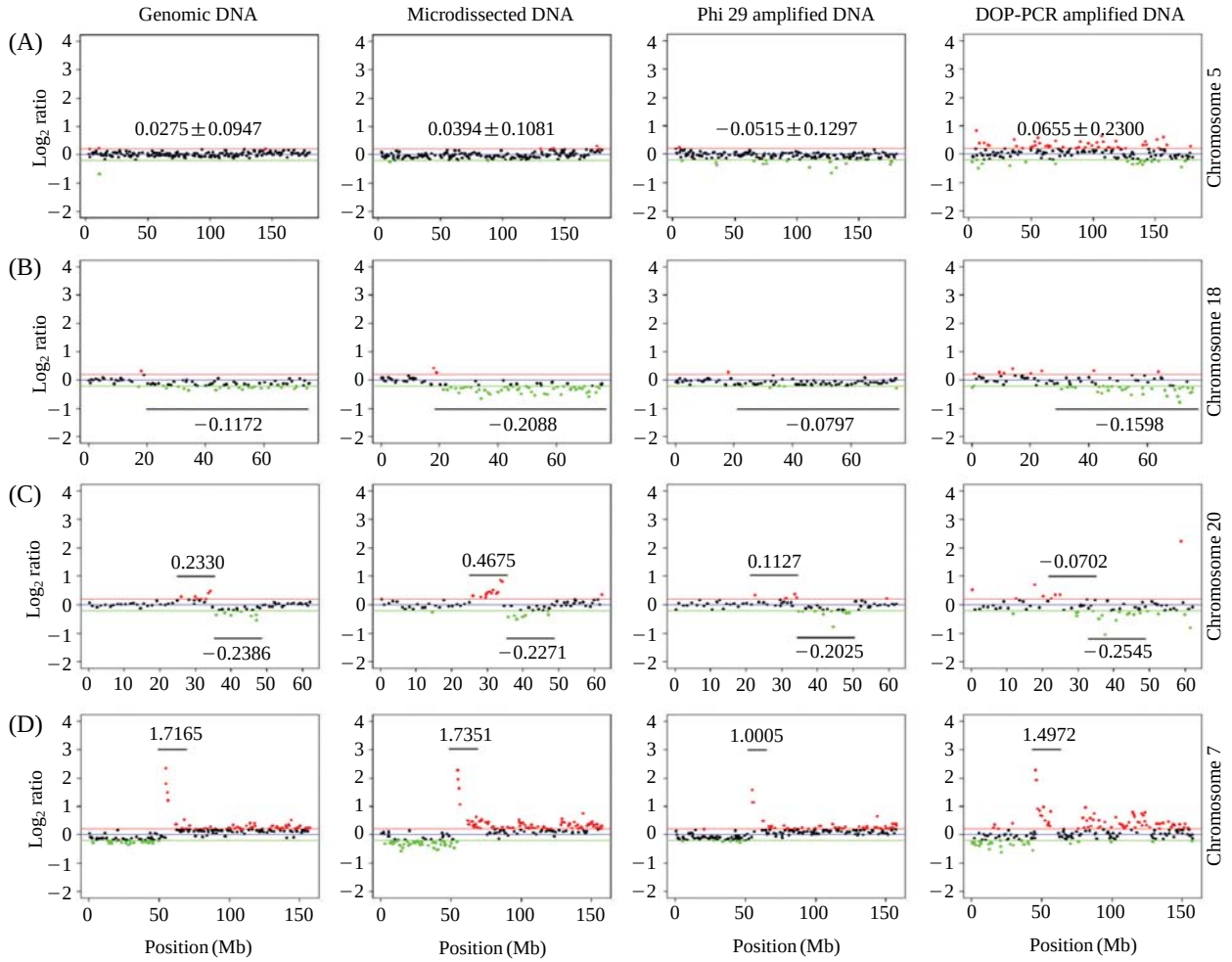

Figure 3. Comparison of performance of array-CGH in 4 different types of DNA; False positive detection rates (FPDR) (A) and sensitivity (B, C, D) by DNA preparation methods. (A) Copy number profiles of the 4 DNA preparation methods in chromosome 5, (B) chromosome 18, (C) chromosome 20 (C), and (D) chromosome 7. X axis represents the linear position on each chromosome (Mb) and Y axis represents the signal intensity ratio (test/reference) in $\log_2$ ratio. Red and green lines represent the cutoffs for copy number changes. Red dot, copy number gain; Green dot, copy number loss; Black dot; no copy number change. Regional copy number alterations are represented as bars with mean signal intensity ratios.

le in both MDA- and DOP-PCR amplified DNAs (Figure 3C). Signal intensity ratios of all 12 clones in this region were above the cutoff level in microdissected DNA, while just 4, 3 and 2 of the 12 clones showed signal intensity ratios above the cut off level in genomic, DOP-PCR amplified and MDA amplified DNA, respectively.

In chromosome 7, there were a 1.05 Mb-sized high-copy amplification on 7p (55.00 to 56.07 Mb) where *EGFR* gene is located, and single copy gain on 7q (Figure 3D). This amplification was clearly detected in all four types of DNA. In both microdissected and genomic DNA, signal intensity ratios (1.74 and 1.72 on average, respectively) were higher than compared to DOP- and MDA-amplified DNA (1.50 and 1.00, respectively). The single copy gain on 7q was also clearly identified in microdissected DNA, while it was less obvious in other type of DNAs.

Discussion

Tumor cell rich genomic DNA is the most important study material for copy number alteration analysis in cancer, especially in poorly demarcated or premaligant lesions. Microdisection is a powerful tool to get tumor cell rich DNA, minimizing the normal cell contamination, which can increase sensitivity/specificity of analysis[6-8]. However, due to the concern over the amount of genomic DNA not enough for whole-

genome analysis, microdissection based array-CGH has not been commonly applied for cancer research in spite of its apparent advantage. Recent advance of microarray technology enabled whole-genome copy number analysis using DNA as small as 400 ng of DNA, which means microdissected DNA can be directly used for array-CGH in many cases. Otherwise, various WGA methods are used to increase the amount of the extracted DNA. However, it has rarely been evaluated whether amplified DNA is suitable for array-CGH. In this study, we evaluated the differences in the results obtained using DNAs prepared in 4 different ways including 2 amplifying methods.

High-level copy number changes such as amplifications on 7p containing *EGFR* and on 3q containing *PIK3CA*, putative lung cancer oncogene, were clearly detected in this study regardless of DNA preparation methods, which is consistent with the previous reports[4,12,13]. However, some low-level copy changes were observed differently according to DNA preparation methods. For example, 53 Mb-sized copy number loss on 18q was clearly detected in microdissected DNA (73%, 44/60 clones), while, in genomic DNA, only 28% of the clones representing according region showed the copy number loss. In DOP-PCR amplified DNA, this alteration were not clearly defined and not detected in MDA-amplified DNA. Our data suggests that both amplified and non-amplified DNA detect high-level copy number changes, and non-amplified, microdissected DNA can detect the low-level copy changes more sensitively. In other word, non-microdissected DNA method showed the lowest FPDR and most sensitive in detecting copy number alterations. Among the amplified methods, DOP-PCR amplified DNA showed higher sensitivity, but similar FPDR to MAD-amplified method. Our data is compatible with the previous comparison of 3 WGA methods for CGH[14], which reported DOP-PCR was the best for whole genome analysis among different types of WGAs.

Our study has several limitations. First, we did not perform microsatellite or STS marker validation to see whether DOP-PCR and MDA amplified products reflect whole genome complexity. However, previous reports have suggested that, in most cases, WGA products represent the genome complexity of original material and copy number profiles can be measured reasonably well with the amplified products, although there is a possibility of false results in a random fashion[12,14,15]. Second, random errors generated during array-CGH hybridization could still remain although theoretically the same hybridization condition was kept, which affected the array profiles. But, in our unpublished data, the false positive rate is usually less than 1% in self-to-self hybridization, which reflects validity of our results.

In conclusion, non-amplified microdissected DNA method showed the best FPDR and sensitivity in detecting copy number alterations in our study. We found the amount of DNA extracted from about 3 sections of 3×3 mm sized lesion was enough to analyze global copy number profiles without WGA. If the amount of DNA is not enough to apply for whole genome array-CGH, DOP-PCR amplification can be a better option than MDA method. Our evaluation will provide useful information for studying copy number alterations, especially based on precancerous and microscopically invaded lesions.

Materials and Methods

Study Materials

Frozen cancer tissue and adjacent normal tissue were obtained from one primary lung cancer patient, who underwent surgical resection at Kangnam St. Mary's Hospital, the Catholic University of Korea. All the procedures including tissue collection and genetic analyses were done under the approval of Institutional Review Board of the Catholic University of Korea.

Tissue Preparation

Paired tumor and adjacent normal tissues were collected from the same patient after surgical resection and snap-frozen in a deep freezer. Genomic DNA was extracted and purified by the standard method[16]. For the microdissection, 20 μm-thick frozen sections were prepared on a gelatin-coated slide using cryotom (Reighert-Jung, St. Gallen, Switzerland). After Hematoxylin & Eosin staining, tumor cell-rich ($>70\%$ of tumor cells) and histologically normal cell areas were selected under the microscope by a board-certified pathologist and dissected manually. Microdissected tissues were transferred into the cell lysis buffer (1% proteinase-K in TE buffer) and DNA was extracted. Extracted DNA was purified using a DNA purification Kit (Solgent, Daejon, Korea) and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Amplification of Microdissected DNA Using Phi 29 DNA Polymerase

GenomiPhi amplification kits (Amersharm Biosciences) were used for the WGA. Ten ng of microdissected DNA was added to 9 μL of sample buffer and heated at 95˚C for 3 min to denature the template DNA. After cooling, 9 μL of reaction buffer and 1 μL

of enzyme mix were added to denatured DNA and incubated at 30˚C overnight. After amplification, Phi 29 DNA polymerase was heat-inactivated at 65˚C.

DOP-PCR Amplification of Microdissected DNA

DOP-PCR primers were prepared according to Fiegler *et al.*'s report[13]: DOP1, 5′-CCGACTCGAG NNNNNNCTAGAA-3′, DOP2, 5′-CCGACTCGAG NNNNNNTAGGAG-3′ and DOP3, 5′-CCGACTCG AGNNNNNNTTCTAG-3′. DOP-PCR reaction was performed as described previously[2,15]. Ten ng of microdissected DNA was used for DOP-PCR. In brief, the reaction was performed using TAPS 2 buffer (250 mM TAPS (pH 9.3), 166 mM $(NH_4)_2SO_4$, 25 mM $MgCl_2$, 0.165% w/v BSA, 5% stock solution; and 0.7 % v/v 2-mercaptoethanol), which is specifically designed for use with Ampli*Taq* polymerase (Perkin-Elmer). The thermal cycling was initiated with a first 3-min denaturation step at 94˚C, followed by 10 cycles of 94˚C for 1.5 min, 30˚C for 2.5 min, ramp at 0.1˚C /sec to 72˚C, 72˚C for 3 min and by 30 cycles of 94˚C for 1 min, 62˚C for 1.5 min, 72˚C 2 min. After 3 different DOP-PCR using DOP-1, 2 and 3, the products were merged for array-CGH.

Array-CGH Hybridization and Data Processing

3K human BAC array covering the entire human genome with 1 Mb resolution was used for profiling genomic alterations[15]. Array-CGH was performed as described elsewhere using MAUI hybridization station (BioMicro Systems, Salt Lake city, UT) (3, 4). Arrays were scanned using GenePix 4100B scanner (Axon Instruments, USA) and the image was processed using GenePix Pro 6.0. Data normalization and re-aligning of raw array-CGH data were performed using web-based array-CGH analysis software ArrayCyGHt (http://genomics.catholic.ac.kr/array CGH/)[17]. We used print-tip loess normalization method for analysis. Linear position of each clone was mapped according to the UCSC genome browser (http://genome.ucsc.edu/; May 2004 freeze).

Determination of Copy Number Alterations

The cutoff value for the copy number alterations was set to be over ± 0.2 in $\log_2$ ratio in this study. A high-level amplification of clones was defined when their intensity ratios were > 1.0 in $\log_2$ ratio, and vice versa for a homozygous deletion. A regional copy number change was defined as DNA copy number alteration limited to a part of a chromosome. The entire chromosome arm gain or loss was determined as previously described[18].

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References

1. Albertson, D. G. & Pinkel, D. Genomic microarrays in human genetic disease and cancer. *Hum Mol Genet* **12**:R145-152 (2003).
2. Chung, Y. J. *et al*. A whole-genome mouse BAC microarray with 1-Mb resolution for analysis of DNA copy number changes by array comparative genomic hybridization. *Genome Res* **14**:188-196 (2004).
3. Kim, M. Y. *et al*. Recurrent genomic alterations with impact on survival in colorectal cancer identified by genome-wide array comparative genomic hybridization. *Gastroenterology* **131**:1913-1924 (2006).
4. Kim, T. M. *et al*. Genome-wide screening of genomic alterations and their clinicopathologic implications in non-small cell lung cancers. *Clin Cancer Res* **11**:8235-8242 (2005).
5. Kang, H. M. *et al*. DNA copy number alterations and expression of relevant genes in mouse thymic lymphomas induced by gamma-irradiation and N-methyl-N-nitrosourea. *Cancer Genet Cytogenet* **166**:27-35 (2006).
6. Cardoso, J. *et al*. Genomic profiling by DNA amplification of laser capture microdissected tissues and array CGH. *Nucleic Acids Res* **32**:e146 (2004).
7. Johnson, N. A. *et al*. Application of array CGH on archival formalin-fixed paraffin-embedded tissues including small numbers of microdissected cells. *Lab Invest* **86**:968-978 (2006).
8. Garnis, C., Coe, B. P., Lam, S. L., MacAulay, C. & Lam, W. L. High-resolution array CGH increases heterogeneity tolerance in the analysis of clinical samples. *Genomics* **85**:790-793 (2005).
9. Hosono, S. *et al*. Unbiased whole genome amplification directly from clinical samples. *Genome Res* **13**:954-964 (2003).
10. Dean, F. B., Nelson, J. R., Giesler, T. L. & Lasken, R. S. Rapid amplification of plasmid and phage DNA using Phi29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Res* **11**:1095-1099 (2001).
11. Dean, F. B. *et al*. Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci USA* **99**:5261-5266 (2002).
12. Massion, P. P. *et al*. Genomic copy number analysis of non-small cell lung cancer using array comparative

genomic hybridization: implications of the phosphatidylinositol 3-kinase pathway. *Cancer Res* **62**:3636-3640 (2002).
13. Shibata, T. *et al*. Genetic classification of lung adenocarcinoma based on array-based comparative genomic hybridization analysis: its association with clinicopathologic features. *Clin Cancer Res* **11**:6177-6185 (2005).
14. Ng, G., Roberts, I. & Coleman, N. Evaluation of 3 methods of whole-genome amplification for subsequent metaphase comparative genomic hybridization. *Diagn Mol Pathol* **14**:203-212 (2005).
15. Fiegler, H. *et al*. DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. *Genes Chromosomes Cancer* **36**:361-374 (2003).
16. Sambrook, J. & Russel, D. W. Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001).
17. Kim, S. Y. *et al*. ArrayCyGHt: a web application for analysis and visualization of arrayCGH data. *Bioinformatics* **21**:2554-2555 (2005).
18. Hackett, C. S. *et al*. Genome-wide array CGH analysis of murine neuroblastoma reveals distinct genomic aberrations which parallel those in human tumors. *Cancer Res* **63**:5266-5273 (2003).