Comparison of Whole Genome Amplification Methods for Further Quantitative Analysis with Microarray-based Comparative Genomic Hybridization

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SUMMARY

Objective: Whole genome amplification (WGA) is a crucial procedure for genomic DNA analysis from limited sources, such as in forensic analysis, embryo biopsy for preimplantation genetic diagnosis, or needle aspiration biopsies. Several strategies for WGA have been developed for either genotyping or microarray-based comparative genome hybridization (array-CGH) during the past decade. Nevertheless, there were few studies in which various WGA methods had been performed side-by-side and results evaluated with multiple methods.

Materials and Methods: Ease of performance, qualitative accuracy, and quantitative fidelity of different WGA methods, such as degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), ligation-mediated PCR (LM-PCR) and strand displacement amplification (SDA), were compared in amplifying genomic DNA derived from karyotype-confirmed amniocytes and the cancer cell line SAOS2.

Results: Using analysis with microsatellite markers, single nucleotide polymorphism markers, and array-CGH, our results suggested that: (1) genomic DNA amplified from DOP-PCR resulted in false positive and negative results by analysis with array-CGH; (2) SDA is the easiest performance method among the three WGA methods; and (3) amplified DNA products generated by LM-PCR best reflect the original genomic DNA.

Conclusion: The amplified DNA products generated by LM-PCR best reflect the original genomic DNA. [*Taiwan J Obstet Gynecol* 2008;47(1):32–41]

Key Words: array-CGH, DOP-PCR, LM-PCR, SDA, whole genome amplification

Introduction

Microarray-based comparative genome hybridization (array-CGH), also known as genomic microarrays [1], is a genome-wide detecting method for imbalanced copy number of DNA sequences [2–4]. Chromosomal alterations can be detected by array-CGH directly, which in turn help us understand the underlying pathophysiology of several diseases such as cancers or congenital syndromes. Nevertheless, the minimal amount of genomic DNA required for hybridization of genomic microarray is about 2–4 μg, and such a DNA amount is still a challenge to obtain from some clinical specimens, such as formalin-embedded tissues, embryo biopsy for preimplantation genetic diagnosis, sperm or oocyte typing, or

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needle aspiration biopsies [5,6]. The available amount of genomic DNA from some clinical specimen is still a limiting factor for genomic analysis with array-CGH.

To overcome the difficulty, several whole genome amplification (WGA) methods have been developed during the past decade, e.g. degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), ligation-mediated polymerase chain reaction (LM-PCR), and strand displacement amplification (SDA) [7]. Each of them has been applied for single nucleotide polymorphism (SNP) analysis, genotyping for short tandem repeat loci, array-CGH analysis, and chromatin immunoprecipitation on chip assay [5,7–9]. Nevertheless, one potential bias of the two thermocycling-based methods (DOP-PCR and LM-PCR) is caused by uneven distribution of GC-rich regions in genomic DNA, which often affects polymerase chain reaction (PCR) amplification [7,10]. SDA is based on the ability of the polymerases, φ29 DNA polymerase and the large fragment of *Bacillus stearothermophilus* (Bst DNA polymerase, large fragment), to cause strand displacement with random amplification initiation points by using random primers [7,10]. Displaced single strands are annealed by new random priming events, and more DNA is generated to form a network of hyperbranched DNA structures [7,10]. Among these WGA methods, SDA has been advocated as the WGA method that possesses the most complete genome coverage and minimal amplification bias [7,10]. However, there were few studies in which various WGA methods had been performed side-by-side and results evaluated with multiple methods.

In this study, we evaluated the ease of performance, qualitative accuracy, and quantitative fidelity of DOP-PCR, LM-PCR and SDA methods in amplifying genomic DNA derived from karyotype-confirmed amniocytes and a cancer cell line. By analysis with microsatellite markers, SNP markers and array-CGH, we compared the efficiency of these methods in representing amplified DNA. Our results suggested that amplified DNA products generated by LM-PCR best reflect the original genomic DNA.

Materials and Methods

Genomic DNA extraction from amniocytes and human cell lines

Four genomic DNAs from three donors with known cytogenetic diagnoses (G-banding) and one cancer cell line were used in this study. Genomic DNA with 46,*XY* was isolated from the peripheral blood of a normal male volunteer, the deletion of 7q34 in a fetus was identified with amniocentesis and chromosomal study in a pregnant woman with balanced translocation t(5;7)(p15.3;q34), and a case of *de novo* inverted duplication of chromosome 10q was diagnosed with both chromosomal G-banding and conventional comparative genome hybridization [11]. The human osteosarcoma cell line, SAOS2, was obtained from the American Type Culture Collection and cultured in DMEM-F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin and 100 units/mL of streptomycin. Genomic DNA was extracted using Puregene DNA isolation kit (Gentra Systems; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA concentration was estimated by spectrophotometry (GeneQuant; Amersham Biosciences, Pittsburgh, PA, USA) and the DNA integrity was confirmed by agarose gel electrophoresis.

Procedures of the three WGA methods

Before being subject to the whole genome amplification procedures, each DNA specimen was diluted to 10 ng/μL with 10 mM Tris–HCl (pH 8.0) containing 0.1 mM EDTA. Ten nanograms of DNA input were used in the following WGA experiments, except that 1 ng of genomic DNA was tested in the SDA experiment.

DOP-PCR

The procedure of the DOP-PCR technique was similar to that previously reported [12], with minor modifications. All thermal cycles were run on a PCR thermocycler (GeneAmp System 9700; Applied Biosystems, Foster City, CA, USA) in two steps. In the preamplification step, 10 ng of both test and reference DNA were used as a template in a 10 μL reaction mixture, which contained 200 μM each of dTTP, dATP, dCTP and dGTP, 1 × Thermosequenase reaction buffer, 1 μM degenerate oligonucleotide primer 5′-CCGACTCGAGNNNNNNAT-GTGG-3′ (Integrated DNA technologies, USA), and 0.4 U/μL Thermosequenase (Amersham Biosciences, Pittsburgh, PA, USA). The four initial cycles were performed at low stringency conditions (denaturation at 94°C for 1 minute, annealing at 30°C for 1 minute, ramp of 0.1°C/sec from 30°C to 72°C, extension at 72°C for 2 minutes, and a final extension of 10 minutes), followed by 35 cycles at high stringency conditions (denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 2 minutes, and a final extension of 10 minutes). AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) was substituted with Thermosequenase in the second step.

LM-PCR

Adaptor ligation-mediated PCR was performed according to a published protocol [13], with slight modifications. In brief, genome DNA was placed in a 55°C heating

block for 10 minutes and digested with 10 U/μL of MseI (New England Biolabs, Ipswich, MA, USA) for 3 hours at 37°C in a volume of 20 μL. After digestion, a total of 25 μL of ligation mixture was prepared on ice and contained 4 mM of MseLig 12 (5′-TAACTAGCATGC-3′), 4 μM of MseLig 21 (5′-ATGGGGATTCCGCATGC-TAGT-3′) and 2.5 μL of 10 × T4 DNA ligase buffer, with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) being added last to prevent self-ligation of the adaptor. In a thermal cycler, the mixture was incubated at 16°C overnight, followed by 70°C for 20 minutes. The volume of the resultant DNA ligation reaction product was brought to 100 μL by adding deionized water. One-tenth volume of the diluted ligated DNA sample was used in the amplification PCR as previously described [13].

SDA

The procedure for the multiple SDA protocol was similar to a previous report [14]. A reaction mixture (10 μL) containing 100 μM of random primer with thiophosphate modification (5′-NpNpNpNpNp^{s}Np^{s}N-3′), 1 × ThermoPol buffer (New England Biolabs, Ipswich, MA, USA) and 4% DMSO was denatured at 95°C for 10 minutes and immediately transferred to ice for 10 minutes. The reaction mixture was then brought to a volume of 30 μL containing 400 μM of dNTPs, 30 ng/μL of T4 gene 32 protein (G32P; Amersham Biosciences, Pittsburgh, PA, USA) and large fragment Bst DNA polymerase (New England Biolabs, Ipswich, MA, USA) at 0.35 units/μL. Reactions were carried out at 50°C for 6 hours. Amplification DNA solution was sonicated with an ultrasonic processor (Misonix, Farmingdale, NY, USA) that was adjusted to generate high output (2 W) for 1-minute pulses. With this setting, DNA fragments would be smaller than 1.5 Kb in size.

Analysis with microsatellite markers

Genotyping analysis was performed with ABI PRISM Linkage Mapping Set version 2.5 (Applied Biosystems, Foster City, CA, USA). For chromosome 7, we selected 18 markers: D7S493, D7S507, D7S515, D7S516, D7S517, D7S519, D7S520, D7S531, D7S630, D7S636, D7S657, D7S661, D7S669, D7S684, D7S798, D7S1870, D7S2465, and D7S2476. For chromosome 8, nine markers (D8S258, D8S260, D8S264, D8S272, D8S277, D8S284, D8S285, D8S549, and D8S1784) were used.

SNP analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) mass spectrometry (MS)

The sample preparation started with a PCR amplification of genomic DNA containing 10 SNP sites. PCR amplification was performed in a final volume of 10 μL containing 20 ng of genomic or amplified DNA, 1 × PCR buffer, 100 μM each of dTTP, dATP, dCTP and dGTP, 1 μM of each primer, and 1 U of Taq DNA polymerase, following by 3 minutes' denaturation at 95°C and 40 cycles of denaturation at 95°C for 30 seconds, annealing at melting temperature of each primer set for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 2 minutes. All thermal cycles were run on a PCR machine (MJ Research, Watertown, MA, USA). The PCR products were treated with magnetic bead purification kit (Genopure ds; Bruker Daltonics, Germany) to remove excess dNTPs and primers. All purification steps were performed on the MAP II/8 robotic workstation (Bruker Daltonics, Germany).

The procedures for each primer extension reaction and SNP analysis by MALDI-TOF MS (Autoflex; Bruker Daltonics, Germany) were as previously reported [15].

DNA array-CGH

Genomic DNA were labeled following a protocol as described by Pollack et al [2]. Two micrograms of sample and reference (46,XX) genomic DNA were labeled with Cy3 and Cy5-dCTP (NEN Life Science Products, Inc., Boston, MA, USA), respectively, using random primers. The labeled probes were hybridized to a human cDNA microarray (Genomic Medicine Research Core Laboratory [GMRCL] Human 15K) containing 15,000 sequence-verified human cDNA clones that have been mapped to 12,530 different genes. Detailed information of GMRCL Human 15K, accession number GPL5354, is available at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL5354). For each pair of specimens, dye swapping was used for minimizing labeling bias and statistical variances of data.

After hybridization and washing, the slides were scanned with a confocal slide scanner, ChipReader (Virtek, Ontario, Canada), to quantify fluorescence intensities, and images were analyzed with GenePix Pro 4.1 software (Axon Instruments, Inc., CA, USA). Data were normalized over the entire microarray by locally weighted linear regression (LOWESS) algorithm, where changes of intensity were assumed to be symmetric for all spots; thus, normalization was performed in each bin of spots. Mapping information and location of all clones were displayed by the order from chromosome 1 to X according to the University of California Santa Cruz genome browser (May 2004 freeze). To evaluate the statistical significance, we performed the one-sample *t* test (null-hypothesis H0, $\mu = 0$) for each 50-gene window. The 50-gene windows were then moved one gene by another along each chromosome. A *p* value of 0.001 was used as the threshold for detecting significant gain or loss of genomic copy number.

All data were calculated with MATLAB 6.5 software (The MathWorks, Inc., MA, USA).

Results

Using 10 ng of genomic DNA as original input, the amplicons for each method were about 0.3 to 4 Kb from DOP-PCR, 0.2 to 2 Kb from LM-PCR, and 0.5 to 20 Kb from SDA, which were similar to previous reports (Figure 1) [7]. The amplicon sizes, ease of performance, and comparison of these three methods are listed in Table 1. To examine if DNA amplification was unbiased during experimental processing, 25 microsatellite markers located on chromosomes 7 and 8 were selected from ABI PRISM Linkage Mapping Set version 2.5 to compare four amplified DNAs with the result of original DNA (2 μg input). As showed in Figure 2, some false negative or positive results were generated during WGA, e.g. a loss of heterozygosity in genotyping for marker D7S517 was found in DOP-PCR amplified DNA that was inconsistent with the result of original DNA (Figure 2). In accordance with the ratio of false positive to negative results, the consistency rate for each WGA method was 67% for DOP-PCR, whereas LM-PCR and SDA showed almost similar consistency rate (~90%). To examine the accuracy for genotyping SNP after WGA, 10 randomly selected SNP markers were genotyped by MALDI-TOF MS to compare with the results from before and after amplification of genomic DNA. The WGA DNA from each method did not show any alterations (homozygosity or heterozygosity) for SNP genotyping as compared with their original DNAs (Figure 3 and Table 2).

To globally survey amplification fidelity of the three WGA methods, the WGA DNAs were applied for

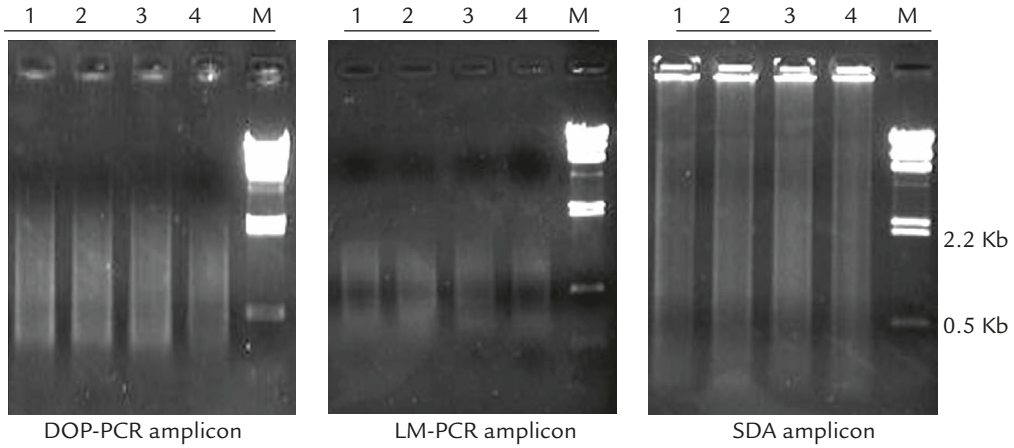

Figure 1. DNA products amplified by three methods of whole genome amplification were visualized on 1% agarose gel stained with ethidium bromide. Lane 1: 46,XY; lane 2: 46,XX,7q34(−); lane 3: 46,XX,10q22(+); lane 4: SAOS2 osteosarcoma cell line; M: λ-Hind III DNA marker. DOP-PCR = degenerate oligonucleotide-primed polymerase chain reaction; LM-PCR = ligation-mediated polymerase chain reaction; SDA = strand displacement amplification.

Table 1. Comparison of three methods for whole genome amplification

	DOP-PCR	LM-PCR	SDA
Pattern	Thermal cycling	Thermal cycling	Isothermal
Product length	0.3–4 Kb	0.2–2 Kb	10–20 Kb
DNA polymerase (Pol)	Thermosequenase and AmpliTaq	Roche Expand Long Template PCR system	Bst polymerase
Error rate of Pol	$1–3 \times 10^{-4}$	4.8×10^{-6}	1×10^{-4}
Time required	< 5 hr	2 days	5 hr
Input DNA			
Reported	12.5 pg*	Single cell (~6 pg)†	10 pg to 10 ng‡
Tested in this study	10 ng	10 ng	1 ng
Ease of performance	Moderate	Least easy	Easiest

**As reported by Huang et al [12]; †as reported by Klein et al [13]; ‡as reported by Honoso et al [30]. DOP-PCR = degenerate oligonucleotide-primed polymerase chain reaction; LM-PCR = ligation-mediated polymerase chain reaction; SDA = strand displacement amplification.*

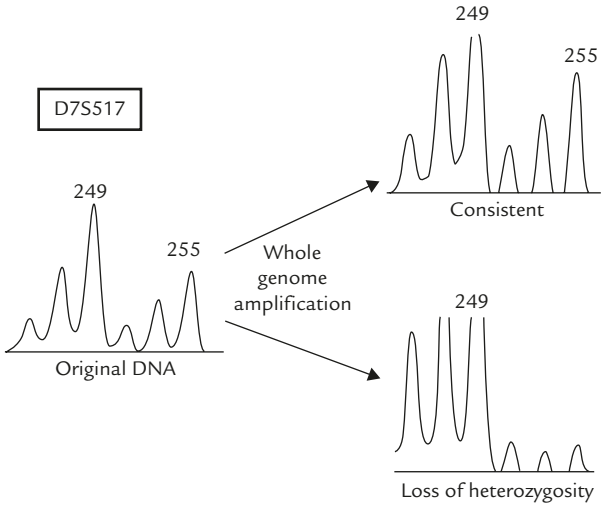

Figure 2. (A) An allele dropout during whole genome amplification was detected as a loss of heterozygosity in genotyping with a microsatellite marker D7S517. (B) Genotyping with 25 microsatellite markers on four DNA samples after whole genome amplification with degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), ligation-mediated polymerase chain reaction (LM-PCR) or strand displacement amplification (SDA) identified that DOP-PCR had the highest error rate and SDA the lowest.

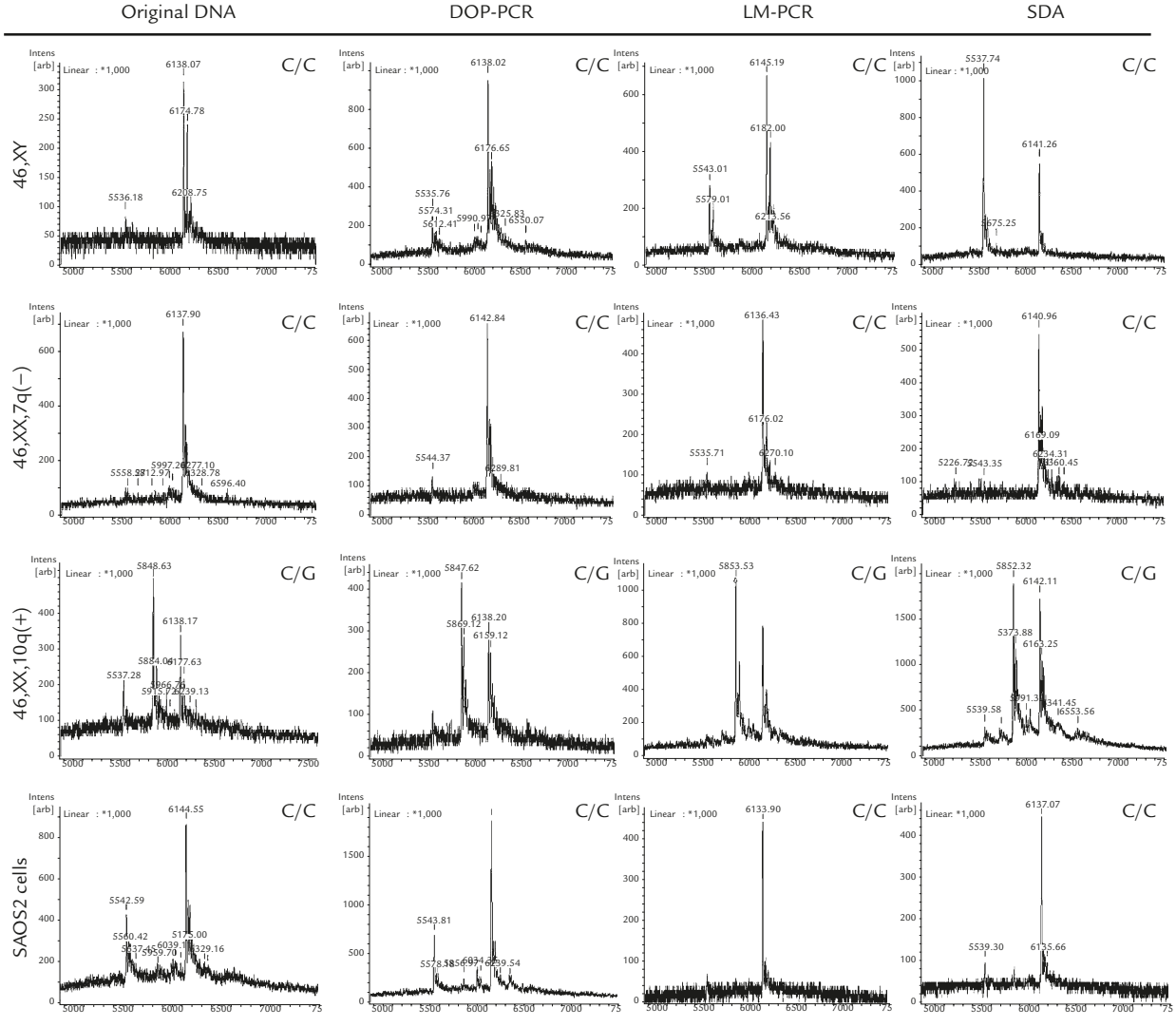

Figure 3. Analysis of a single nucleotide polymorphism (SNP) marker in CYP1B1(rs0010012) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. DOP-PCR = degenerate oligonucleotide-primed polymerase chain reaction; LM-PCR = ligation-mediated polymerase chain reaction; SDA = strand displacement amplification.

Table 2. Comparison of the three whole genome amplification (WGA) methods by using single nucleotide polymorphism (SNP) markers

SNP ID	Chromosome allelic variation	Concordance with genomic DNA (%)		
		DOP-PCR	LM-PCR	SDA
rs0449856	A/T	100	100	100
rs0010012	G/C	100	100	100
rs2020874	G/T	100	100	100
rs2066479	A/G	100	100	100
rs4646422	A/G	100	100	100
rs8191246	A/G	100	100	100
(Tumor necrosis factor)	C/T	100	100	100
(Transforming growth factor-beta)	C/T	100	100	100
rs605059	C/T	100	100	100
rs9340773	A/G	100	100	100

DOP-PCR = degenerate oligonucleotide-primed polymerase chain reaction; LM-PCR = ligation-mediated polymerase chain reaction; SDA = strand displacement amplification.

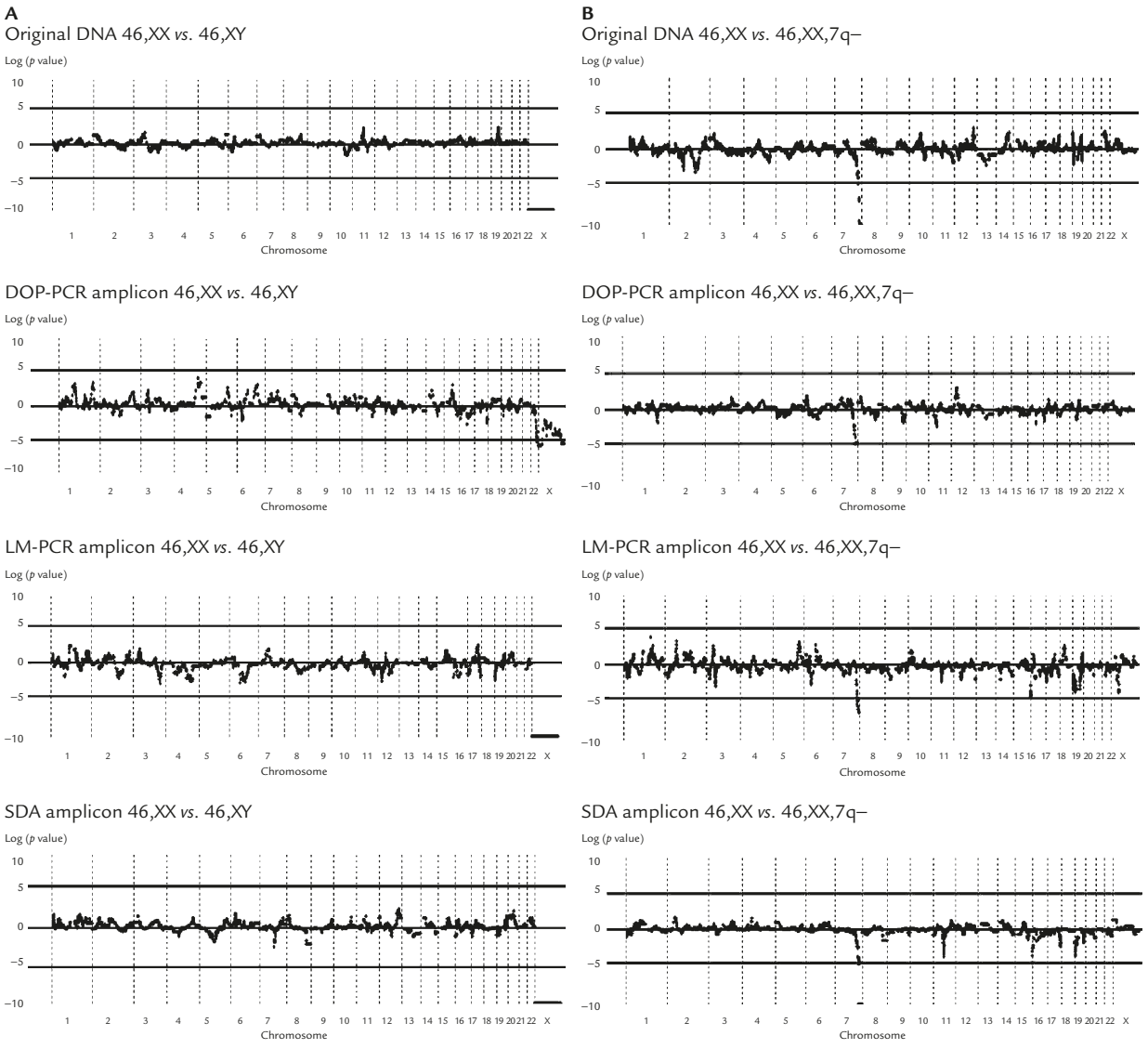

Figure 4. *Continued*

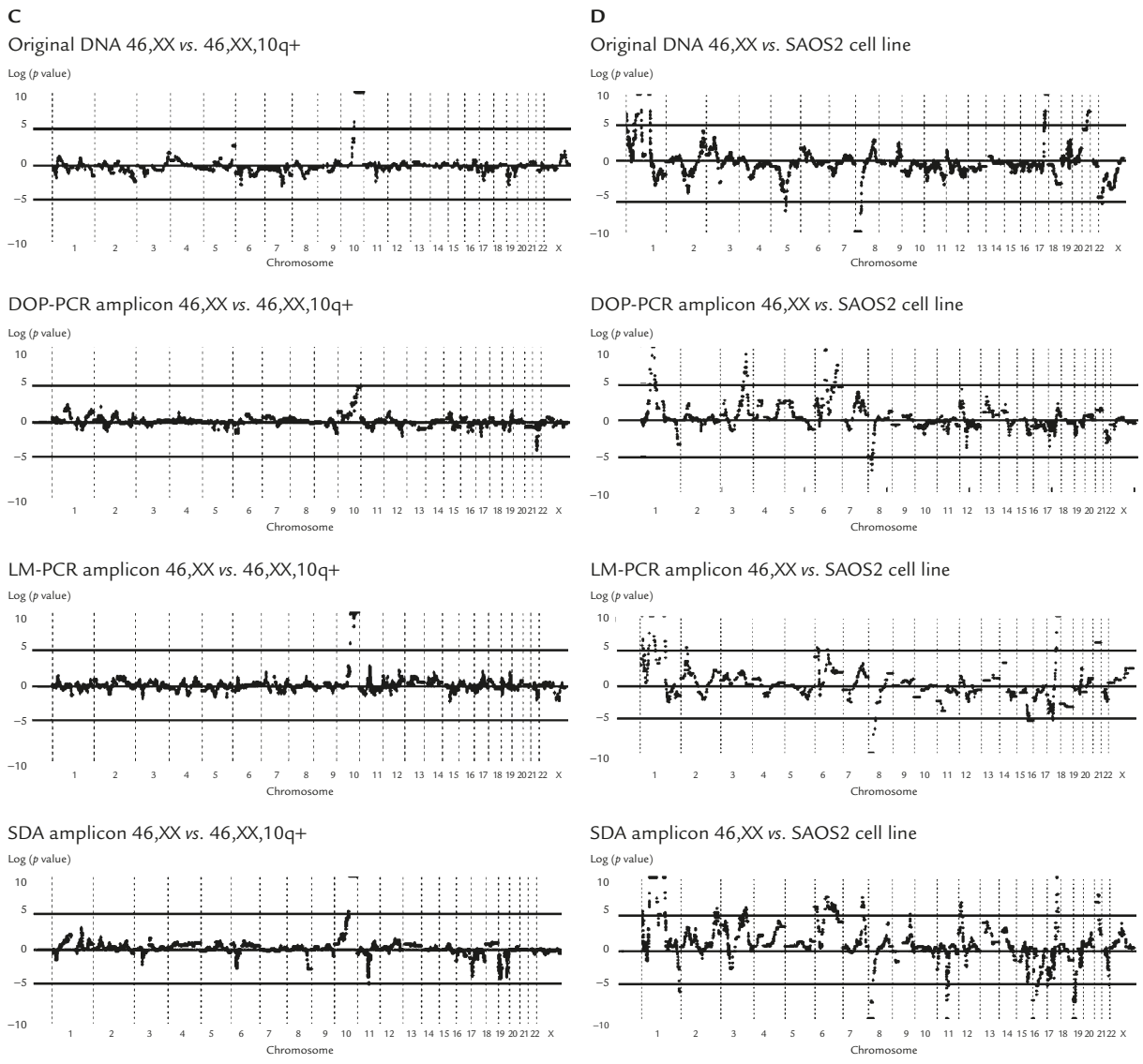

Figure 4. Comparison of microarray-based comparative genome hybridization between the original DNA and each amplified DNA generated by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), ligation-mediated polymerase chain reaction (LM-PCR) and strand displacement amplification (SDA) on specimens obtained from: (A) 46,XY; (B) 46,XX,7q–; (C) 46,XX,10q+; and (D) SAOS2 osteosarcoma cell line. The karyotype of the reference DNA was 46,XX.

quantitative analysis with array-CGH and the results were also compared with the result of original genomic DNA. As shown in Table 3, loss of statistical significance (false negative) and artificial addition of statistical significance (false positive) were examined in the 46,XY, 46,XX,7q–, 46,XX,10q+ and SAOS2 cells when amplified by each WGA method (Figure 4 and Table 2). According to results of array-CGH, amplification with DOP-PCR tended to induce false negative results; whereas both false negative and false positive amplifications were generated by all three WGA methods, especially in SAOS2 cancer cell lines, which were not generated by the LM-PCR or SDA method in the 46,XY, 46,XX,7q– and 46,XX, 10q+ samples. Among these WGA methods, amplified DNA products generated by LM-PCR best reflect the original genomic DNA by analysis with array-CGH.

All three WGA methods yielded sufficient DNA products for subsequent studies, such as microsatellite genotyping, SNP genotyping and array-CGH, when 10 ng of genomic DNA were used as input. When time and ease of procedure were considered, DOP-PCR and SDA were much better than LM-PCR. Evaluation with 10 SNP markers did not detect any mutation that had been introduced during any of the three WGA methods. Genotyping with 25 microsatellite markers, however, revealed that DOP-PCR generated the highest error rate at 33%, with a similar tendency shown with results of array-CGH. Therefore, our results suggested LM-PCR to be the most reliable among the three WGA methods. When time and

Table 3. Comparison of microarray-based comparative genome hybridization between the original DNA and each amplified DNA generated by degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), ligation-mediated polymerase chain reaction (LM-PCR) and strand displacement amplification (SDA)

G-banding	Original DNA (2 mg)	DOP-PCR (10 ng)	LM-PCR (10 ng)	SDA (10 ng)
46,XY	46,XY	46,XY	46,XY	46,XY
46,XX,7q(−)	46,XX,7q(−)	46,XX	46,XX,7q(−)	46,XX,7q(−)
46,XX,10q(+)	46,XX,10q(+)	46,XX	46,XX,10q(+)	46,XX,10q(+)
SASO2	Amp: 1p, 1q, 17q, 21	Amp: 1p, 1q, 3q, 6q	Amp:1p, 1q, 17q, 21	Amp: 1p, 1q, 17q, 21, 6q
	Del: 8q		Del: 8q	Del: 8q, 11p, 16p, 19p

Amp = amplification; Del = deletion.

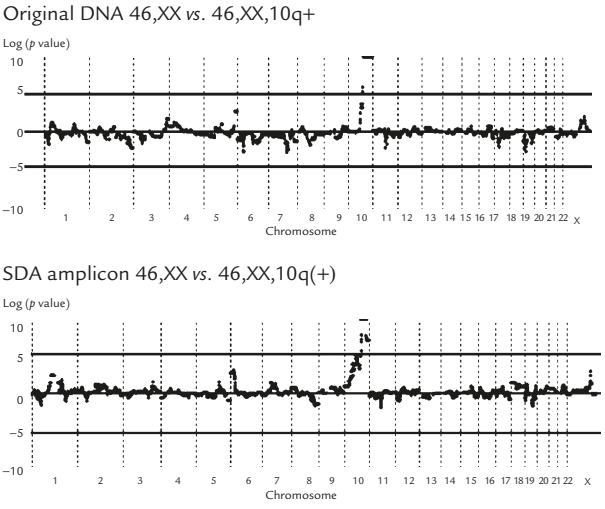

Figure 5. Two examples of successful strand displacement amplification (SDA) with 1 ng input of genomic DNA isolated from amniocytes with karyotype 46,XX,10q+.

ease of performance were considered, SDA was the easiest method among these WGA methods. Previous reports have reported success in amplification of trace amount of genomic DNA (10 ng) by SDA; therefore, to test the limitation of SDA, we further examined the efficiency of SDA by using 1 ng of genomic DNA as original input [14]. As evaluated with array-CGH, the genomic profile of 46,XX,10q+ after SDA with 1 ng input of genomic DNA was identical to that of the original DNA (Figure 5).

Discussion

Geneticists have employed chromosome banding, various types of fluorescence *in situ* hybridization, and conventional CGH for genetic testing. With the advantages of high resolution and high throughput, array CGH has been advocated as the new cytogenetics [16]. Although its performance, applications and data interpretation remain to be validated in clinical applications, array-CGH provides a whole genome analysis in one hybridization reaction for detecting the changes of genomic DNA copy number. To further extend the applications of array CGH on any clinical specimens that may contain only nanogram levels of genomic DNA, such as the cells isolated by laser-capture microdissection [17], the addition of a reliable WGA, however, appears indispensable. Vice versa, the nature of array-CGH in whole-genome coverage lends itself as the most critical method for validating the qualitative and quantitative performance of WGA [14].

Classified by the sequence length of DNA clones on the slides, the three types of array-CGH used are those with genomic clones (usually larger than 50,000 bp) [18–21], cDNA clones (500 to 3,000 bp) [2,3,22], and oligonucleotides (20 to 100 nucleotides) [23,24]. The advantages and limitations of the three platforms have been reviewed by Mantripragada et al [1], which will not be discussed here. One critical factor in determining the reliability of microarray results is the signal-to-noise ratio of microarray hybridization experiments [1]. Signal-to-noise ratios are usually determined by the efficiency of fluorescent labeling and detection and the background reading of slide surface. In this study, we used GMRCL Human 15K cDNA microarrays for array-CGH analysis, in which the choices of slide and spotting solution have been thoroughly verified [25].

The advantages of spotting cDNA clones on microarrays for genomic analysis include: (1) many cDNA clone sets are available worldwide [3,22,25–28], and (2) cDNA microarrays provide the same platform for doing parallel analysis of changes in DNA copy number and expression levels using the same set of genes [1]. This type of parallel analysis has correlated with an upregulated gene expression to 62% of amplified genes in human primary breast tumors [3] and has led to the identification of genes important in the tumorigenesis of many tumors [22,28,29]. On the other hand, because only exons of genes and expressed sequence tags can be analyzed with cDNA microarrays, the drawbacks of

using cDNA microarray for array CGH analysis include: (1) an uneven distribution of measurement points across the genome; and (2) the inability to analyze the roles of gene regulation by promoters, introns, and intergenic sequences in gene regulation.

When we used 10 ng of genomic DNA as input to compare DOP-PCR, LM-PCR, and SDA, all three methods yielded sufficient DNA products for subsequent molecular studies, such as microsatellite genotyping, SNP genotyping and array-CGH. When time and ease of procedure were considered, DOP-PCR and SDA were much better than LM-PCR. Evaluation with 10 SNP markers did not detect any mutation that had been introduced during any of the three WGA methods. However, genotyping with 25 microsatellite markers revealed that DOP-PCR generated the highest error rate (33%). Collectively, with the evaluation with array-CGH, our results suggested that LM-PCR is the most reliable among the three WGA methods and SDA is the easiest method to amplify genomic DNA.

In one reaction, SDA can be performed directly from clinical specimens (whole blood, dried blood, buccal cells, culture cells, buffy coats) without prior DNA isolation [30] and can thereby be easily automated with a robotic liquid handler. These attributes lend SDA a promising role in high-throughput processing. Two enzymes, Bst and φ29 polymerases, have been used in SDA [14,30,31], accounting for the priming event in SDA reaction being propagated over very long distances (> 20 Kb) in the genome. The error rate of φ29 DNA polymerase (1 in 10^5 to 10^6 bp) [32] is much lower than that of Taq polymerase (3 in 10^4 bp) [33]. The error rate of Bst DNA polymerase (1 in 10^4) is higher than φ29 DNA polymerase; but for equal amplification of DNA along the genome with SDA, Bst DNA polymerase was shown to be better than φ29 polymerase in preserving the original genomic dosage [14]. Finally, the disadvantages of SDA are the higher costs for thiophosphate-modified degenerate primers and Bst DNA polymerase than the two PCR-based methods.

In conclusion, array-CGH provides the only method to scan the whole genome for relative dosage alteration in genomic DNA before and after WGA. Combined with the high-fidelity WGA with LM-PCR method, array-CGH can have many clinical applications, such as embryo preimplantation genetic diagnosis, prenatal diagnosis and cancer research.

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