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Evaluation of the Reliability of Chromosomal Imbalances Detected by Combined Use of Universal DNA Amplification and Comparative Genomic Hybridization

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Comparative genomic hybridization (CGH) analysis of microscopic tumor samples is allowed by universal DNA amplification using degenerate oligonucleotide primed-PCR (DOP-PCR). To evaluate the reliablity of DOP-PCR CGH, we performed DOP-PCR CGH and standard CGH in parallel using DNAs extracted from 10 malignant tumors of the hepatobiliary tract and pancreas. Similar results were obtained by both methods with a few exceptions, indicating that DOP-PCR CGH provides cytogenetic information equivalent to that obtained from standard CGH. We also investigated the sensitivity of DOP-PCR CGH using sequential dilutions of DNA from microdissected tumor cells. DOP-PCR using 100 to 800 pg of template DNA yielded successful CGH results. However, less than 50 pg of template DNA was not suitable because of the small amount of generated DNA. These findings suggest that DOP-PCR CGH is applicable for CGH analysis of tiny specimens which are too small for standard CGH. Accordingly, DOP-PCR CGH analysis may become a useful method in clinical laboratory examination.

Key words: Microdissection — Degenerate oligonucleotide primed-PCR (DOP-PCR) — Comparative genomic hybridization (CGH)

Comparative genomic hybridization (CGH) is a useful molecular cytogenetic technique for genome-wide screening of chromosomal imbalances.[1)] In a single hybridization, CGH allows detection and mapping of relative DNA copy number increases or decreases across the whole genome. However, the sensitivity of CGH is decreased by normal cells contaminating the tumor samples.[2,3)] Application of a microdissection technique increases the sensitivity because the target cells can be collected more precisely, and this is especially critical for CGH analyses of solid tumors which contain stromal components.[4,5)] However, as a result of microdissection, the sample size for CGH analysis becomes small. In tiny specimens from biopsy or cytology, it is hard to obtain enough DNA for standard CGH because it requires 0.5 to 1 μg of genomic DNA, corresponding to approximately 50 to 100 thousand diploid cells.[1)]

Telenius *et al.*[6,7)] developed degenerate oligonucleotide primed-PCR (DOP-PCR), which allows universal amplification of target DNA. CGH analysis of tumor cells can therefore be performed by incorporating DOP-PCR. Regarding solid tumors, there are several reports of genetic aberrations analyzed by CGH combined with DOP-PCR, so-called DOP-PCR CGH.[8–16)] However, to our knowledge, only a few studies have been done to examine the validity of this method.[17–19)] In those studies, materials were limited to cell lines and whole blood, which contain no stromal components. However, when DOP-PCR CGH analysis is adopted for clinical laboratory examination in the near future, the samples will be clinical samples, such as surgical specimens and/or specimens from biopsy and cytology. It still remains to be evaluated whether DOP-PCR can universally amplify all sequences of template DNA for CGH, especially in clinical materials. Accordingly, we set out to evaluate the reliability of DOP-PCR CGH using 10 surgically removed malignant tumors of the hepatobiliary tract and pancreas, in comparison with standard CGH. In addition, the minimal amounts of DNA required for DOP-PCR were studied by means of sequential dilutions of DNA from microdissected cells.

MATERIALS AND METHODS

Tumor samples In this investigation, 10 specimens were obtained from 10 surgically removed malignant tumors of the hepatobiliary tract and pancreas (Table I). Of these, three tumors were hepatocellular carcinomas (HCCs), which were histologically well, moderately, and poorly differentiated, respectively. Two tumors were gallbladder cancers, two were bile duct cancers, two were pancreatic cancers, and one was cancer of the ampulla of Vater. All samples were stored at −80°C until use.

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Tissue preparation Six to seven serial frozen tissue sections (20 μm thick) were prepared. An additional 4-μm section was also made for detailed histologic examination. All slides were stained with hematoxylin-eosin to identify cancerous regions.

Microdissection and DNA extraction Tumor fragments were microdissected from tissue sections with a sterile 26-gauge needle under an inverted microscope (Nikon 66906, Nikon, Tokyo) using a 10× objective lens and were collected into a microtube. Then, genomic DNA was extracted using a DNA extraction kit (SepaGene, Sankoujunyaku Co., Ltd., Tokyo) according to the manufacturer's instructions. As a control, reference DNA was isolated from normal peripheral blood lymphocytes. A portion of each extracted DNA was diluted with distilled H_2O to 11 to 34 ng/μl (Table I), and 1 μl of the diluted DNA was used as template for DOP-PCR amplification.

DOP-PCR DOP-PCR was performed using universal primer 6-MW (5′-CCGACTCGAGNNNNNNATGTGG-3′) on a thermocycler (ASTEC, Fukuoka) as previously described, but with slight modifications.[7, 18, 20] Briefly, a 1 μl aliquot of microdissected DNA was added to 4 μl of 1× Sequenase buffer and pretreated with 1 U Topoisomerase-I (Promega, Madison, WI) for 30 min at 37°C. The Topo-I pretreatment was followed by five cycles of Thermosequenase (20 U) (Amersham, Cleveland, OH) treatment (1 min at 94°C, 2 min at 30°C, and 2 min at 37°C). Preamplification was followed by one cycle at 95°C for 10 min, and 45 μl of 1× PCR buffer with 2.5 U of *Taq* DNA polymerase (TaKaRa, Shiga) was then added. This was followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min, with a final extension at 72°C for 5 min. Each PCR run included samples of normal genomic DNA and a blank to check contamination. Concentration and size of each PCR product was determined by fluorometric measurements (Hoefer DQ200, Amersham) and 1% agarose gel electrophoresis, respectively. Microdissected DNA yielded up to 5.5 μg of PCR product that averaged 1300 bp in size (range, 400 bp to 4 kb).

DNA labeling Extracted DNAs (0.5 μg) from tumor tissues and reference normal cells were labeled with SpectrumGreen-dUTP and SpectrumRed-dUTP (Vysis Inc., Downers Grove, IL) by nick translation, respectively.

CGH and digital image analysis (DIA) CGH analysis, including digital image analysis, was carried out as previously described.[1, 2] Each labeled DNA sample (200 ng) and 10 μg of Cot-1 DNA (Gibco BRL, Gaithersburg, MD) were dissolved in 10 μl of hybridization buffer and co-hybridized onto normal denatured metaphase chromosomes for 48 h at 37°C. The specimens were mounted in an anti-fade solution containing 0.15 mg/ml 4′,6-diamino-2-phenylindole as a counterstain. Images were captured with an Olympus BX 50 epifluorescence microscope equipped with a 100× UplanApo objective and a CCD camera (SenSys 1400, Photometrics Ltd., Tucson, AZ). The DIA system ("QUIP" XL, Vysis Inc.) developed specifically for CGH was used in this experiment. At least 10 representative images were analyzed, and the results from these were combined to produce an average fluorescence ratio for each chromosome. Increases and decreases in DNA sequence copy number were defined by tumor/reference ratios of >1.2 and <0.8, respectively. High-level copy number increases in subregions (amplifications), in contrast to a whole-arm gain, were defined by a tumor/

Table I. Summary of Microdissected Fresh-frozen Tissue Sections of Tumor

No.	Age/Sex	Type of tumor[a)]	Histology[b)]	UICC stage	DNA conc.[c)] (ng/μl)	DNA conc. after dilution (ng/μl)	Amount of DNA after dilution (ng)	Fold amplification
1	65/M	HCC	HCC, por	IVa	399	23 (17.3)[d)]	5000	217
2	72/M	HCC	HCC, well	III	470	21 (22.4)	4700	224
3	56/M	HCC	HCC, mod	II	607	24 (25.3)	5100	213
4	70/F	GB ca	adeno, por	IVb	324	34 (9.5)	4400	129
5	69/M	GB ca	adeno, mod	IVa	175	21 (8.3)	5400	257
6	51/F	BD ca	adeno, mod	IVa	273	20 (13.7)	5300	265
7	53/F	BD ca	adeno, mod	IVa	137	11 (12.5)	5050	459
8	78/F	Panc ca	adeno, mod	IVb	80	25 (3.5)	4550	182
9	47/M	Panc ca	adeno, mod	IVa	268	27 (9.9)	4500	166
10	62/F	Vater ca	adeno, por	III	720	27 (26.7)	4250	157

a) HCC, hepatocellular carcinoma; GB ca, gallbladder cancer; BD ca, bile duct cancer; Panc ca, pancreatic cancer; Vater ca, cancer of the ampulla of Vater.
b) adeno, adenocarcinoma; mod, moderately differentiated; por, poorly differentiated.
c) DNA conc., DNA concentration.
d) ( ), dilution rate.

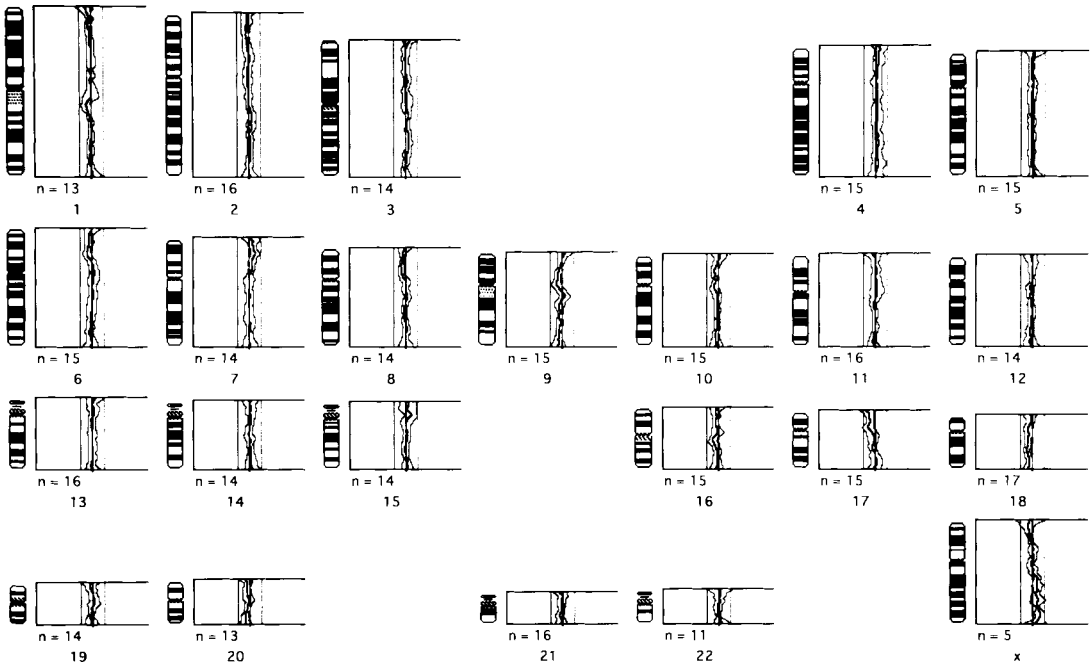

Fig. 1. CGH fluorescence intensity profiles between DOP-PCR-amplified normal DNA labeled with SpectrumGreen and normal DNA labeled with SpectrumRed. The green-to-red fluorescence ratios are presented. No ratio changes are seen along any of the chromosomes. The mean ratio (thick line)±1 SD (thin lines) is plotted. These profiles were confirmed by "inverse" CGH.

reference ratio of >1.4. Each CGH experiment included a normal SpectrumGreen-labeled DNA sample from a healthy woman as a negative control, and a SpectrumGreen-labeled MPE-600 breast cancer cell line DNA sample as a positive control. The cut-off values described earlier were determined from the negative control hybridizations as well as from positive specimens. In the negative control hybridizations, the mean green/red ratio and the standard deviation stayed between 0.8 and 1.2 along all of the chromosomes. In the positive controls, for which the MPE-600 cell line was used, known aberrations did not differ between the cut-off values of 0.75 and 1.25, and 0.8 and 1.2.

RESULTS

Negative control As a control study, we performed CGH using DOP-PCR-amplified normal DNA labeled with SpectrumGreen and normal DNA labeled with SpectrumRed. Uniform and even hybridizations were obtained for all chromosomes (Fig. 1). A similar result was also obtained from "inverse" CGH.[21)]

DOP-PCR CGH Following dilution of samples to the concentrations shown in Table II, we performed DOP-PCR with our protocol. Electrophoresis by 1% agarose gel revealed that PCR products appeared as a smear with sizes ranging from 400 bp to 4 kb. This was similar to the result of a previously reported study.[18)] The final yields consisted of 4250 to 5400 ng of DNA per sample (Table I).

Comparison of DOP-PCR CGH with standard CGH Standard CGH and DOP-PCR CGH results are shown in Table II. CGH profiles from the two methods were similar (Fig. 2), although there were four cases with a different region. Namely, in DOP-PCR CGH profiles, loss of 4q21–q32 and gain of chromosome 19 appeared in cases 3 and 6, respectively, whereas loss of 1p and gain of 7p14–pter were absent in cases 4 and 8, respectively (Fig. 3, A).

Sensitivity of DOP-PCR CGH We also investigated the sensitivity of DOP-PCR CGH using microdissected cells from well-differentiated HCC (case 1 in Table I). Based on DNA concentration, sequential dilutions of DNA (5, 15, 50, 100, 200, 400, 600, and 800 pg) were made and used as template for DOP-PCR amplification (Fig. 4, A). PCR from more than 100 pg of template DNA yielded 4.5 to 5.5 μg of final products. DOP-PCR CGH analysis of these yielded profiles identical to those obtained with a standard CGH protocol. However, less than 50 pg of template DNA did not yield sufficient amplification products. Since one diploid cell contains approximately 6.6 pg of genomic DNA in the nucleus, this means that DOP-PCR can amplify DNA from 15 copies of diploid genome, but not from less than 7 copies.

Table II. Comparison of DOP-PCR CGH with Standard CGH in Total Cases

No.	Method[a)]	Chromosomal imbalances[b)]
1	(S)	1q22–qter (+), 6p21–pter (–), 6p21 (+), 7 (+), 8p (–), 8q23–qter (+), 12p (–), 15q (+), 17p (–)
	(D)	1q22–qter (+), 6p21–pter (–), 6p21 (+), 7 (+), 8p (–), 8q23–qter (+), 12p (–), 15q (+), 17p (–)
2	(S)	1q (+), 2 (+), 4q (–), 5q14–q23 (–), 8p (–), 8q (+), 9 (–), 13q (–), 15q (–), 16 (–), 17p (–), 19 (–), 21q (–)
	(D)	1q (+), 2 (+), 4q (–), 5q14–q23 (–), 8p (–), 8q (+), 9 (–), 13q (–), 15q (–), 16 (–), 17p (–), 19 (–), 21q (–)
3	(S)	8p (–), 8q (+), 9p13–p22 (–), 11q14 (+), 16p (–)
	(D)	4q21–q32 (–), 8p (–), 8q (+), 9p13–p22 (–), 11q14 (+), 16p (–)
4	(S)	1p (–), 5q (–), 7p (+), 10q25–qter (–), 15q (–), 17p (–), 17q (+), 21q (–)
	(D)	5q (–), 7p (+), 10q25–qter (–), 15q (–), 17p (–), 17q (+), 21q (–)
5	(S)	5p14–pter (+), 5q12–q22 (–), 13q (–), 18p (+), 18q21–q22 (–)
	(D)	5p14–pter (+), 5q12–q22 (–), 13q (–), 18p (+), 18q21–q22 (–)
6	(S)	3q12–q13 (–), 3q26–qter (+), 5 (–), 6p12–p21 (+), 6q15–q25 (–), 9p (–), 14q13–q24 (–), 17q24–qter (+), 18q (–), 20q (+)
	(D)	3q12–q13 (–), 3q26–qter (+), 5 (–), 6p12–p21 (+), 6q15–q25 (–), 9p (–), 14q13–q24 (–), 17q24–qter (+), 18q (–), 19 (+), 20q (+)
7	(S)	1p (–), 1q25–q31 (+), 4 (–), 6 (–), 12q15–qter (–), 18 (–)
	(D)	1p (–), 1q25–q31 (+), 4 (–), 6 (–), 12q15–qter (–), 18 (–)
8	(S)	4q (–), 7p14–pter (+), 8q24–qter (+), 17p12–pter (–), 18q (–), 22q (+), Xq26–qter (+)
	(D)	4q (–), 8q24–qter (+), 17p12–pter (–), 18q (–), 22q (+), Xq26–qter (+)
9	(S)	8q23–qter (+), 9p (–), 17p (–), 18q12–q22 (–), 19q (+), 22q (+), Xq21–qter (–)
	(D)	8q23–qter (+), 9p (–), 17p (–), 18q12–q22 (–), 19q (+), 22q (+), Xq21–qter (–)
10	(S)	4q21–q24 (–), 5p14–pter (+), 5q13–q15 (–), 9 (–), 13q (–), 14q31–qter (+), 17p (–), 18 (–), Xq21–qter (–)
	(D)	4q21–q24 (–), 5p14–pter (+), 5q13–q15 (–), 9 (–), 13q (–), 14q31–qter (+), 17p (–), 18 (–), Xq21–qter (–)

a) (S), standard CGH; (D), DOP-PCR CGH.
b) (+), gain; (–), loss.

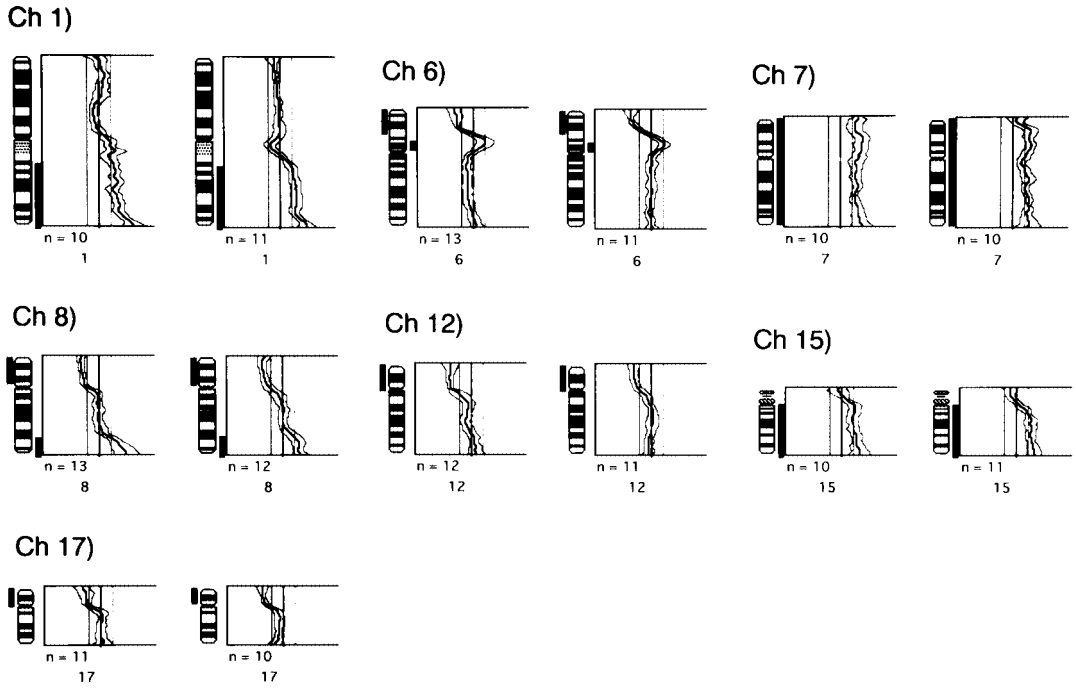

Fig. 2. CGH profiles of standard CGH and DOP-PCR CGH for case 1 (left and right, respectively). Gains are shown on the right side of the chromosome ideograms and losses on the left side. Both profiles were similar. In Ch *n*), *n* is the chromosome number.

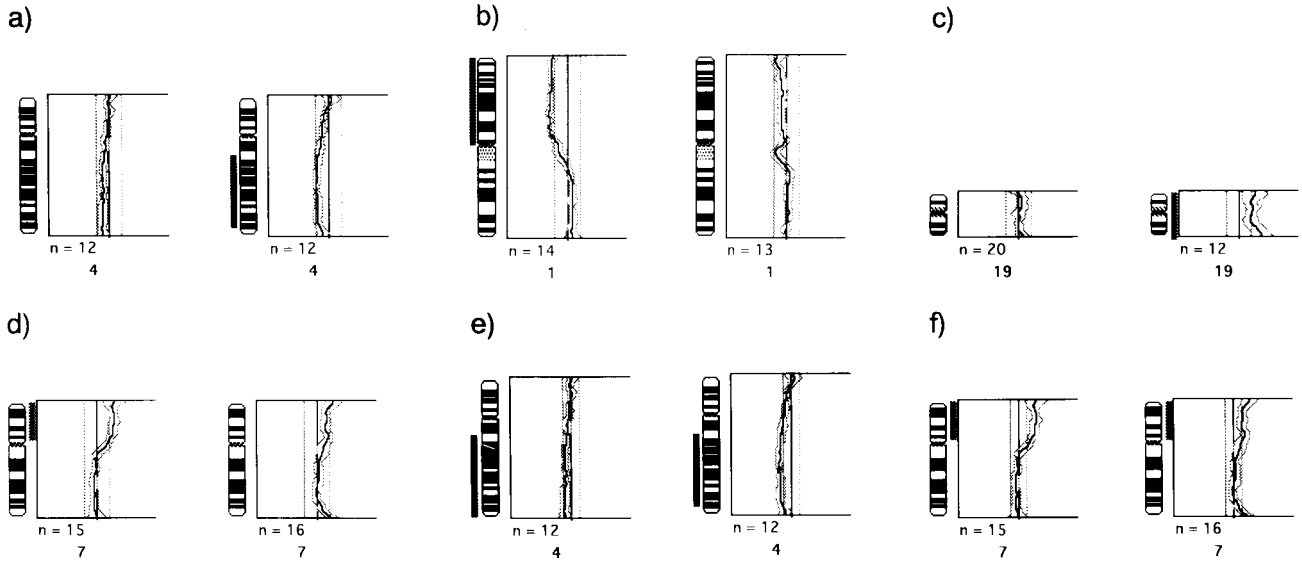

Fig. 3. Comparison of DOP-PCR CGH profiles with those of standard CGH in four cases with a different region. CGH profiles of standard CGH (left) and DOP-PCR CGH (right). (A) The discrepant results are shown: a) chromosome 4 in case 3, b) chromosome 1 in case 4, c) chromosome 19 in case 6, d) chromosome 7 in case 8. (B) Comparison of CGH profiles with a different cut-off value (0.83–1.17). Similar results for both methods were obtained by adjusting the cut-off values to 0.83 and 1.17. This may have been due to small differences in hybridization to the metaphase spreads used. e) Chromosome 4 in case 3, f) chromosome 7 in case 8.

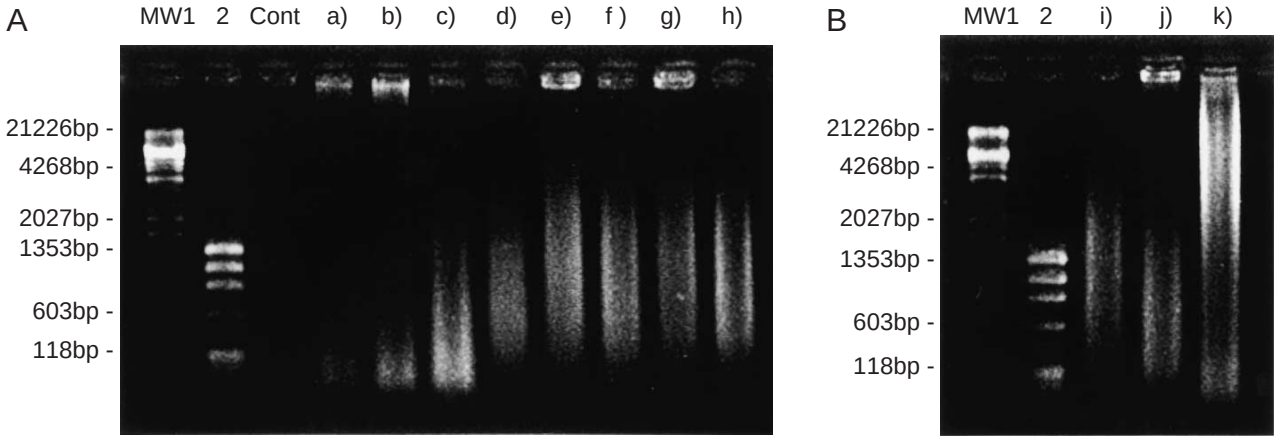

Fig. 4. Ethidium bromide-stained 1% agarose gel showing the results of DOP-PCR amplification. (A) Sequential dilutions of DNA from one case of HCC were used as templates for DOP-PCR amplification. Products from more than 100 pg of template DNA occur in a smear ranging from 400 bp to 4 kb in size. However, less than 50 pg of template DNA did not yield sufficient products. MW, molecular weight markers; Cont, no template DNA. a) 8, b) 15, c) 50, d) 100, e) 200, f) 400, g) 600, h) 800 pg of template DNA. (B) Two characteristics of poor quality PCRs. i) A successful case, j) too short PCR products, k) non-specific PCR products.

DISCUSSION

In 10 malignant samples, the final products of DOP-PCR consisted of 4250 to 5400 ng of DNA and were not related to the initial amount of template DNA. It was expected that the amounts would reach a plateau state by 35 cycles during the second PCR step. *Taq* polymerase used in this step has no proofreading function. Since it is well known that *Taq* polymerase tends to convert GC to AT and causes small deletions and point mutations, the products may include misincorporated nucleotides due to replication error (RER).[12] However, it is known that

probes including RER will cohybridize to suitable loci of the metaphase spread. Therefore, these minimal changes are not expected to affect DOP-PCR CGH results.

We compared CGH profiles from the two methods. There were four cases with an inconsistent region. Of these, chromosome 19 and 1p reflected the well-known uncertainty of CGH analysis on these loci.[2)] Thus, we should cautiously evaluate alterations of these loci in DOP-PCR CGH, as well as in standard CGH. Chromosomes 4q21–q32 and 7p14–pter showed similar results with both methods when the cut-off values were adjusted to 0.83 and 1.17 (Fig. 3, B). This may have been due to small differences in hybridization to the used metaphase spreads, since the quality of CGH analysis depends considerably on that of the metaphase spreads.

There were two characteristics of poor quality PCRs (Fig. 4, B). One problem was the formation of too short PCR products. Lengths of less than 600 bp were observed and these very short probes yielded insufficient hybridization in most cases. In rare cases, successful CGH results can be obtained from these probes without modification of the nick translation conditions. The other problem was non-specific PCR products. This was caused by the use of too much *Taq* polymerase in the second PCR step. Appropriate-length products were obtained by reducing the amount of *Taq* polymerase.

DOP-PCR CGH provides cytogenetic information equivalent to that obtained from standard CGH. Since it allows CGH analysis of tiny specimens which are too small for standard CGH, it should become a useful method in clinical laboratory examination.

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