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SINGLE CELL DEGENERATE OLIGONUCLEOTIDE PRIMER-PCR AND COMPARATIVE GENOMIC HYBRIDIZATION WITH MODIFIED CONTROL REFERENCE

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Abstract: For investigating the possibility of applying degenerate oligonucleotide primer PCR (DOP-PCR) and comparative genomic hybridization (CGH) technique to analyses of genomic genetics in a single cell, the whole genomic DNA of a single cell with XX, XY, XO, XXY, +13 or +21 was amplified by DOP-PCR. Single cell DOP-PCR CGHs with conventional and modified control references, the genomic DNA and a single cell DOP-PCR product from normal male, were carried out respectively. The results showed that the average profile of the fluorescence intensity ratio in CGH with the genomic DNA as reference fluctuates much and that the standard deviation in about 30% haploid is beyond the normal limits. False positive hyper-representation was found to exist in X chromosome while trisomy 13 and 21 were not detected. However, the distributions of the mean and the standard deviation of the ratio in the CGH with DOP-PCR product as reference were quite acceptable. The copy number changes of chromosome X, Y, 13 and 21 were revealed. Those results suggested that there is unrandom unequal amplification in a single cell DOP-PCR. Using a single DOP-PCR product as reference can decrease its influence on CGH. Single cell DOP-PCR-CGH and its application in the genetic analyses of preimplantation embryo or fetal cell in maternal blood may be possible.

Key words: degenerate oligonucleotide primer PCR; comparative genomic hybridization; single cell
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INTRODUCTION

Fluorescence in-situ hybridization (FISH) and polymerase chain reaction (PCR) had been successfully used for preimplantation genentic diagnosis (PGD) (Verlinsky et al., 1997, Sabtalo et al. 1995) although they normally only offer information from one or few chromosome regions or specific loci. Degenerate oligonucleotide primer -PCR (DOP-PCR) technique was designed for general amplification of whole genomic DNA at frequently occurring priming sites of the degenerate oligonucleotide primer under conditions of low annealing temperature and two steps of amplification (Cheung et al., 1996). Comparative genomic hybridization (CGH) is a technique for determining the gains and losses of chromosome copy number by means of labeling the test and control DNA with different color fluorochrome, reverse hybridization, and comparison of the two different fluorescence intensities (Kallioniemi, 1992). The advantages of application of DOP-PCR and CGH to PGD had been discussed (Van-den-Veyver et al., 1998, Harper et al., 1996,). The research in this field has just started, so only two reports have been published so far (Wells et al., 1999, 2000).

For the investigation of the possibility of using DOP-PCR-CGH to analyze the whole genome in single cell level, the study of single cell DOP-PCR CGHs with conventional and modified control references were carried out respectively.

MATERIALS AND METHODS

Single cell DOP-PCR The single lymphocyte from individuals with karyotype 46, XY, 46, XX, 45, XO, 47, XXY, 47, XY, +13, and 47, XY, +21 was sorted into 0.5 ml PCR and lyzed according to Cui et al.(1995) separately.

For DOP-PCR, the following solutions were added into each tube: 5 μl of 20 μmol/L degenerated universal primer (5'-CCG ACT CGA GNN NNN NAT GTG G-3') (Boeheinger Mannheim); 0.01% gelatin; 100 mmol/L Tris-HCl, pH 8.3; 20 mmol/L each dNTP; 20

mmol/L $MgCl_2$ and 1 μl of Taq polymerase (Perkin-Elmer/Cetus, 5 units/μl). Final reaction volume was 50 μl.

DOP-PCR was run on a thermocycler (Minicyler, MJ Research) in initial denature at 94℃ for 10 min, 5 cycles of random amplification: 94℃ for 1 min, 30℃ for 1.5 min, 30-72℃ for 3 min, 72℃ for 3 min; 35 cycles of nonrandom amplification: 94℃ for 1 min, 62℃ for 1 min, 72℃ for 3 min + 3 sec/cycle. DOP-PCR efficiency was checked in 1 % agarose gel. DOP-PCR products were purified by high purity PCR purification kits (Boeheinger Mannheim) and the DNA concentration was measured in spectrophotometer.

DNA labeling The DOP-PCR product was labeled with SpectrumGreen-dUTP (Vysis) or TRITC-dUTP (Boeheinger Mannheim) by nick translation (Kuukasjarvi et al., 1997). The length of the labeled DOP-PCR product was checked in 1 % agarose gel.

CGH 400 ng of SpectrumGreen labeled DOP-PCR product (SG-DOP-PCR), equal amount of SpectrumRed total human male genomic reference DNA (Vysis) (XY-SR-DNA) or TRITC-labeled DOP-PCR product from single normal male lymphocyte (XY-TRTIC- DOP-PCR) and 10 μg of human Cot1 DNA (Promega) were combined and precipitated in 1/10 volume of 3 mol/L sodium acetate and 2 volumes of 100% ethanol. Cot1 DNA was applied for blocking the variable repeated heterochromatic regions (Kallioniemi 1992). CGH was prepared according to Kallioniemi et al. (1994).

CGH images were caught by a CCD camera using the Quips XL Genetics Workstation (Vysis 30 – 14 320) or Cytovision Ultra image collection system (Applied Imaging Int Ltd) and CGH analyses were carried out by Quips CGH analysis software (Vysis 30 – 143 002). For each CGH test, 5 metaphases were analyzed. The distributions of the green to red fluorescence intensity ratios in each metaphase were profiled. The average profile and its standard deviation in each CGH were displayed. The ratios of the standard deviations exceeding 0.85 – 1.15 in normal autosome regions in the two kinds of CGHs were compared by T-test.

RESULTS

1. DOP-PCR product 30 single lymphocytes were amplified by single cell DOP-PCR (5 cells for each case). The size of the single cell DOP-PCR product ranged from 150 – 3000 bp with about 2.0 μg of total DNA according to the spectrophotometer measurement after purification. The negative control always contained primer-related products with size of less than 1000 bp.

2. Fluorochrome labeled product By means of adjusting the concentration ratio of DNA polymerase I and DNAse I in nick translation, the size of the SpectrumGreen-dUTP or TRITC labeled DOP-PCR products were controlled within the range of 100 – 1500 bp.

3. CGH analyses 30 CGHs of SpectrumGreen labeled DOP-PCR product from a single cell with above 6 karyotypes against SpectrumRed total human male genomic reference DNA (SG-DOP-PCR/ XY-SR-DNA CGH) and 30 against TRITC-labelled DOP-PCR product from single normal male lymphocyte (SG-DOP-PCR/ XY- TRITC-DOP-PCR CGH) were analyzed. Excluding the data on the centeromeric, telemetric, and other heterochromatic regions and the short arms of the acrocentric chromosomes, the results were as follows.

SG-DOP-PCR/XY-SR-DNA CGH (1) There were significant variations of average profiles of the green to red fluorescence ratios and in about 30 % of normal autosome regions, the standard deviations exceeded 0.85 – 1.15 (Table 1), the acceptable range for CGH analyses (Kallioniemi et al., 1994). (2) The X chromosomes were over-representative (> 1.15) even in XO or XY although the intensity ratios increased with the X copy number. (3) A slight increase tendency of the trisomy 13 profile was seen, but it still remained within the normal threshold of 0.85 – 1.15 (Fig. 1). No over-representation was found in trisomy 21.

Table 1 Comparison of the ratios of the standard deviations exceeding 0.85 – 1.15 in the two kinds of CGHs

CGH	*n*	Mean	SD	χ^2	*P*
SG-DOP-PCR/XY-SR-DNA	30	0.063	0.026	17.526	< 0.01
SG-DOP-PCR/XY-TRITC-DOP-PCR	30	0.317	0.075		

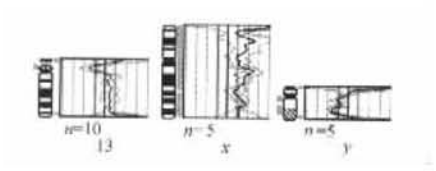

Fig.1 DOP-PCR product from a single 47, XY, + 13 cell / normal male genomic DNA CGH

There are significant variations of average profiles of the green to red fluorescence ratios and the X chromosome is false overrepresentive (> 1.15). A slight increase tendency of the profile in trisomy 13 is seen, but it still remains within the normal threshold of 0.85 – 1.15.

SG-DOP-PCR/XY- TRITC-DOP-PCR CGH (1) The distributions of average profiles in normal autosome regions were close to 1.0. The regions with standard deviations exceeding 0.85 – 1.15 comprised only 6.3 % of autosomic regions and were significantly fewer than those in SG-DOP-PCR/ XY-SR-DNA CGHs (Table 1). (2) The X-chromosome profiles were located about 1.0 in XY or XO and were over-representative (> 1.15) in XX and XXY. The Y chromosome ratios were under-representative (< 0.85) in XO and XX. (3) There was over-representation of chromosomes 13 and 21 in trisomy 13 (Fig.2) and 21 (> 1.15).

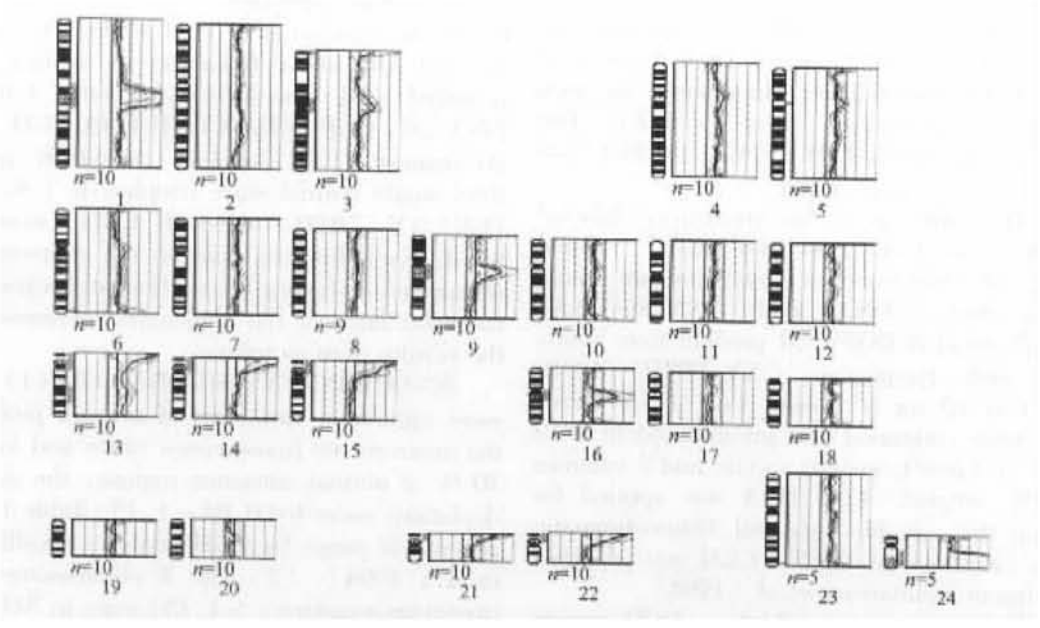

Fig.2 DOP-PCR product from a single 47, XY, + 13 cell / normal male single cell DOP-PCR product CGH

The distributions of average profiles in normal autosome regions are close to the expectation, 1.0. The variations of the average profiles and the standard deviations are acceptable. The profiles of X and Y-chromosomes are located in about 1.0. There are the overrepsentatives of chromosome 13 (> 1.15).

DISCUSSION

CGHs with amplified whole genomic DNA by DOP-PCR had been successfully applied to genetic analysis of small amount tissue (Kuukasjarvi et al., 1997). However, since there is a trend toward lower amplificability of the DOP-PCR product with lower starting genomic DNAs, the results of DOP-PCR-CGH with genomic DNA diluted to single cell level were unsatisfactory (Kuukasjarvi et al., 1997; Telenuis et al., 1992). In the present study, a single cell DNA was directly used as the template. The more than 2.0 μg purified products obtained after DOP-PCR was enough for more than 3 CGH tests. The results suggested that the amplification efficiency of single cell DOP-PCR was quite high.

For CGH test, the uniformity of amplification is more important than the efficiency. In the SG-DOP-PCR/ XY-SR-DNA CGH, normal genomic DNA was used as the reference control DNA. The results reflected the amplification uniformity of single cell DOP-PCR along the genome.

The average profile of the fluorescence intensity ratio obviously fluctuated, 1/3 of standard deviations exceeded the normal range, X chromosome was in false-positive over-representative and the detection of trisomy 13 and 21 was failed. All of those suggested that single cell DOP-PCR could not uniformly amplify the whole genome completely. Using conventional control reference, normal genomic DNA to perform CGH of single cell DOP-PCR product would increase false positive or false negative risks.

However, when we modified CGH control system by applying DOP-PCR product from a single normal male lymphocyte as control reference to SG-DOP-PCR/ XY- TRITC-DOP-PCR CGH, the variations of the average profiles were significantly decreased. Most of the distributions of the standard deviations were within the normal range and false over-representation of X chromosome disappeared. The CGH reflected the copy number of X and Y chromosome and showed the over-representation of chromosomes 13 and 21 in trisomy 13 and 21 respectively. These results suggested that the amplification fluctuation of the single cell DOP-PCR could be unrandom and that using DOP-PCR product as control reference could significantly reduce its influence on CGH. Single cell DOP-PCR-CGH can not only detect the copy number change of relatively longer chromosomes, such as X and 13, but also possibly find duplication and loss of the relatively shorter chromosomes such as chromosomes 21 and Y. Single cell DOP-PCR-CGH and its application for genetic study of preimplantation embryo or fetal cell in maternal blood may be possible.

It should be pointed out that even in CGH with DOP-PCR product from a single normal male lymphocyte as control reference, about 6% of standard deviations of the average profiles still exceeded the normal range. The profiles in the partial autosomic regions of chromosome 13 and 21 in trisomy 13 and 21 did not show over-representation. Causes for those phenomena might be associated with the low level of starting DNA, unequal exposure of the priming points and DNA primer-related product (Telenuis et al., 1992). One thermocycle condition and use of various kinds of primers during DOP-PCR could be another possible reason. Therefore, modifying the methods of single cell lysis, or even DOP-PCR primers, may be helpful for further improvement of single cell DOP-PCR-CGH technique. Further study on the sensitivity of single cell DOP-PCR-CGH in detecting the partial chromosome abnormality must be done before its application to the whole genome study of preimplantation embryo or fetal cell in maternal blood.

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