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# Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridisation

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## ABSTRACT

**Molecular genetic analysis of isolated single cells and other minute DNA samples is limited because there is insufficient DNA to perform more than one independent PCR amplification. One solution to this problem is to first amplify the entire genome, thus providing enough DNA for numerous subsequent PCRs. In this study we have investigated four different methods of whole genome amplification performed on single cells, and have identified a protocol that generates sufficient quantities of DNA for comparative genomic hybridisation (CGH) as well as more than 90 independent amplification reactions. Thus, numerous specific loci and the copy number of every chromosome can be assessed in a single cell. We report here the first reliable application of CGH to single cells from human preimplantation embryos (blastomeres) and to single fibroblasts, buccal cells and amniocytes.**

Procedures such as preimplantation genetic diagnosis, analysis of fetal cells isolated from the maternal circulation or cervical canal, forensic science and investigation of ancient DNA are performed on minute quantities of DNA or even single cells. For molecular analysis of such small samples it is essential to first amplify the DNA using the polymerase chain reaction (PCR). However, the original sample (DNA template) cannot be efficiently retrieved following PCR and consequently the amount of information gained is limited to that which can be obtained in a single amplification reaction. Multiplex PCR protocols have been employed for the simultaneous amplification of as many as 15 individual loci (1); however, design of compatible sets of primers can be problematic and it may be difficult to distinguish between the different DNA fragments produced.

Cytogenetic studies of small numbers of cells are equally fraught, with a low probability of any given cell being sampled during metaphase. Cell culture to generate metaphase chromosomes requires living cells and also a significant length of time. Moreover, current techniques are relatively inefficient at the single cell level. Multicolour fluorescent *in situ* hybridisation (FISH) performed on interphase nuclei for the detection of specific chromosomal regions has overcome some of these problems, circumventing the need for cell culture (2). However, technical difficulties limit the number of chromosomes that can be accurately assessed with few studies using more than five probes.

We have investigated methods that allow more genetic information to be obtained from minute DNA samples, and from single cells in particular. These methods aim to generate a non-specific amplification of all genomic sequences [whole genome amplification (WGA)]. Using such techniques a single genome can be amplified to provide sufficient DNA templates for a number of subsequent PCR amplifications. Furthermore, WGA products can be returned to at a later date, allowing new tests or confirmatory diagnosis to be performed.

As well as enhancing PCR-based analysis, WGA also has the potential to increase the amount of cytogenetic information that can be obtained from samples of minute DNA content. This is dependent on sufficient DNA being generated for comparative genomic hybridisation (CGH) analysis, a technique related to FISH which, in a single hybridisation, allows the relative copy number of all 23 pairs of chromosomes to be determined (3).

The WGA techniques assessed during this study were degenerate oligonucleotide primed PCR (DOP-PCR; 4), tagged PCR (T-PCR; 5), primer extension preamplification (PEP; 6) and alu PCR (7). Initial experiments were conducted on single fibroblasts, buccal cells, amniocytes and embryonic cells isolated by micromanipulation, and lysed as reported previously (8). WGA protocols were optimised by alteration of a variety of reaction parameters including concentrations of primers, nucleotides and *Taq* polymerase, and by adjusting thermal cycling conditions: full methodological details of all protocols are available upon request.

It was essential to maintain stringent precautions against contamination (9) throughout single cell isolation, lysis and amplification procedures. The incidence of contamination was assessed regularly using numerous control blanks containing PCR reaction mixture but no DNA. Twenty-five single cells were amplified using each WGA method. All WGA reactions were conducted in a 50 µl volume containing PCR buffer (HT Biotechnology) and a lysed single cell. Two DOP-PCR protocols were tested (DOP25 and DOP50) and in each case reaction mixtures contained 0.2 mM deoxynucleoside triphosphates (dATP, dTTP, dCTP, dGTP), 2.0 µM DOP primer (4) and 2.5 U

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*Taq* polymerase (HT Biotechnology). Thermal cycling conditions were as follows: 94°C for 9 min; 94°C for 1 min, 30°C for 1.5 min and 72°C for 3 min (eight cycles); 94°C for 1 min, 62°C for 1 min and 72°C for 1.5 min (25 or 50 cycles; DOP25 and DOP50, respectively); 72°C for 8 min. For DOP25, 5 µl of the reaction product (1/10 vol) was removed and subjected to further amplification in a fresh microcentrifuge tube containing PCR buffer, 0.2 mM dNTPs, 2.0 µM DOP primer and 2.0 U *Taq* polymerase in a total volume of 50 µl. The mixture was then heated to 94°C for 4 min followed by 25 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 1.5 min and a final incubation at 72°C for 8 min. During the second DOP25 reaction it was possible to label the DNA for CGH; in this case only 0.1 mM dTTP was included in the reaction mixture and 2.5 µl of fluorescein-11-dUTP or rhodamine-4-dUTP (Amersham) was added.

The proportion of the genome efficiently amplified by the different WGA techniques was tested by amplification of 10 different loci from 4 µl aliquots taken from each WGA product (Table 1) using quantitative fluorescent PCR (QF-PCR). Primer sequences, concentrations and thermal cycling programmes were as described previously (8). The WGA products and negative controls were amplified for 35 cycles while the positive controls of 8 ng of reference genomic DNA underwent 25 cycles. Fluorescent PCR products were separated and analysed using automated laser DNA analysers [Perkin Elmer ABI Prism 310 and Pharmacia Automated Laser Fluorescence (ALF)] using protocols reported by Sherlock and co-workers (8). This allowed specific PCR products to be sized and the amount of each amplicon evaluated by the extent of fluorescent activity.

**Table 1.** Genomic coverage: proportion of successful amplifications at specific loci after various forms of WGA

| Locus | WGA method | | | | | |
|---|---|---|---|---|---|---|
| | PEP | T-PCR | DOP25 | DOP50 | alu | Total |
| D21S11 | 1.00 | 0.88 | 0.73 | 0.80 | 0.41 | 0.77 |
| D21S1414 | 0.90 | 0.65 | 0.76 | 0.85 | 0.45 | 0.76 |
| D18S535 | 0.90 | 0.69 | 1.00 | 0.95 | 0.80 | 0.86 |
| CFTR | 0.95 | 0.95 | 0.74 | 1.00 | 0.86 | 0.92 |
| β-globin | 0.90 | 0.94 | 0.86 | 0.91 | 0.52 | 0.85 |
| FACC | 0.80 | 0.41 | 0.81 | 0.91 | 0.19 | 0.64 |
| Amg X | 0.91 | 0.82 | 0.95 | 0.80 | 0.47 | 0.80 |
| Amg Y | 0.90 | 0.50 | 0.80 | 0.80 | 0.67 | 0.73 |
| APC | 0.95 | 0.91 | 0.91 | 0.95 | 0.80 | 0.94 |
| HMSH2 | 0.90 | 0.87 | 0.89 | 0.91 | 0.72 | 0.86 |
| Total | 0.91 | 0.76 | 0.85 | 0.89 | 0.59 | 0.81 |

Twenty-five isolated single cells were amplified by each WGA method and tested for each locus.

The PEP and DOP50 methods provided the most complete coverage of the genome with 228/250 (91%) and 223/250 (89%) of loci successfully amplified, respectively. Alu PCR performed worst with only 148 of the 250 amplifications (59%) being successful after WGA. Previous studies have reported similar amplification efficiencies after PEP and no evidence of amplification bias (6,10); however, other reports have suggested that preferential annealing sites for DOP primers may exist (4). Significant differences in the amplification efficiencies of different loci were noted after alu PCR and T-PCR suggesting that these methods amplify certain genomic sequences more efficiently than others. The distribution of alu repeats throughout the genome is not uniform and consequently a range of amplification efficiencies (0.19 for FACC to 0.86 for CFTR) had been anticipated. The variation in amplification efficiencies observed subsequent to T-PCR (0.41–0.95) was less expected, but could possibly be overcome by redesigning the primer. Our data suggests that neither alu PCR nor the T-PCR protocol used in this study can be used reliably in conjunction with molecular analyses of single cells.

No significant difference between PEP employing random primers 15 or 16 nt in length was observed. Differences between DOP25, involving 25 cycles of amplification after which a 5 µl aliquot of DOP-PCR product was removed and subjected to a further 25 cycles of amplification, and DOP50, consisting of 50 consecutive cycles of amplification all conducted in the same tube, were also negligible. DOP25 had the advantage that after the first 25 cycles, 10 separate aliquots could be taken and used to set up further DOP25 reactions. Multiple independent amplifications of the adenomatous polyposis coli (APC) gene demonstrated that together the 10 reactions produce sufficient DNA from a single cell for at least 90 PCR amplifications.

Single cell amplification using DOP-PCR, alu PCR and T-PCR produced a much greater quantity of DNA than PEP. It was possible to visualise the heterogeneous mixture of fragments generated by these techniques as a smear on 1% agarose gels stained with ethidium bromide. This was not possible using PEP amplification. Interestingly, several distinct bands could be seen within the smear produced by T-PCR, again indicating that amplification using this technique might not be entirely random. No correlation between the size of amplified fragments (ranging from 100 to 450 bp) and PCR efficiency was observed suggesting that all methods have no difficulty in producing fragments of up to 450 bp. Moreover the existence of amplified fragments >1500 bp in length could be demonstrated by gel electrophoresis of DOP-PCR, T-PCR and alu-PCR products. Total failure of WGA occurred at a similar frequency for each method investigated (4–8%) and in most cases was probably the result of loss of the cell during the isolation procedure.

Regardless of the WGA method used, there was little difference in the amplification efficiency of microsatellite loci when compared with that of unique sequences; however, the fidelity of replication of the former was poor. A large number of PCR artefacts, particularly small deletions and insertions were seen to affect all repetitive loci. Allele sizes were usually increased or decreased by a number of base pairs equivalent to one repeat length, although in some cases several repeat units were deleted or inserted. This phenomenon appears to be a consequence of WGA since direct amplification of microsatellite loci from isolated single cells did not give fragments of unexpected size (8). The phenomenon displayed some locus specificity possibly due to variation in factors such as chromatin structure, GC content and whether the repeat is perfect or disrupted by other sequences. The most likely cause of these artefacts is replication slippage exacerbated by the low annealing temperatures that are characteristic of all the WGA techniques investigated. Preheating reactions to 94°C before the addition of *Taq* (hot-start) was not found to improve the fidelity of any WGA method. Errors of microsatellite amplification have been previously reported after the analysis of small quantities of DNA. Focault and co-workers had observed similar errors when

$(CA)_n$ repeats were amplified from quantities of DNA less than 10 cells (11); however, not all authors have encountered such difficulties with direct amplification of single cells. Cheung and Nelson reported compatibility of DOP-PCR amplification with accurate amplification of di-, tri- and tetranucleotide repeats from 40 ng DNA samples (12). However, application of their reaction conditions to single cells (10 pg) produced the same artefacts as we had observed previously.

In addition to changes in the length of microsatellite sequences, QF-PCR revealed that the two alleles of a heterozygous locus are not necessarily amplified to an equal extent (up to 30-fold differences were observed). In some cases amplification of one of the two alleles failed altogether. This phenomenon, known as allele drop-out (ADO), is frequently observed after the amplification of DNA fragments from single cells (13). All WGA methods displayed similar levels of ADO, which affected 3–5% of unique sequences (e.g. CFTR ΔF508 carrier cells). Equal amplification of both alleles was observed for 17–40% of heterozygous microsatellite loci, depending on which WGA method was used. This contrasts to 78% of microsatellite alleles equally amplified directly from single cells (8). Like ADO, preferential amplification affected alleles of a given locus at random. Amplification of several aliquots of the same WGA product revealed similar errors in all reactions suggesting that mistakes during the replication of microsatellites, preferential amplification of alleles and ADO occurred during the initial WGA reaction rather than during the subsequent PCR. A consequence of unequal amplification of alleles was that attempts to detect aneuploidy in trisomy 21 and trisomy 18 single cells using QF-PCR, as reported for larger DNA samples (14), were seldom successful.

Specific genomic fragments amplified from WGA products were subjected to a variety of mutation analysis techniques to test the compatibility of these protocols with WGA. Three primers were used simultaneously to detect the sickle cell mutation in an adapted amplification refractory mutation system (ARMS) procedure, as previously reported (8). Single strand conformation polymorphism (SSCP) and heteroduplex analysis were employed for the detection of mutations in CFTR (ΔF508 deletion), β-globin (sickle cell, A to T substitution) and polymorphism in the APC and hMSH2 genes (G to A and C to T changes, respectively). These analyses were carried out using the Phastsystem™ (Pharmacia) using protocols described previously (15). Heteroduplex analysis and SSCP, which rely on the physical properties of the amplified DNA strand, were shown to be entirely compatible with all forms of WGA. All the mutations and polymorphisms were successfully detected. However, incorrect results were obtained in ~5% of cells amplified by PEP or DOP and then analysed for the sickle cell mutation using ARMS. Mutant and normal allele-specific products were detected in 3/50 homozygous normal cells amplified by PEP methods and 2/50 normal cells amplified by DOP-PCR. However, in both cases QF-PCR revealed that the spurious mutant product was only present at a fraction of the concentration of the normal product.

For cytogenetic analysis of single cells using CGH it was first necessary to label the WGA products. Fluorescent labelling could be achieved using nick translation kits (Boehringer Mannheim). Typically, 1 μg of DNA was treated according to the manufacturer's instructions. Alternatively, the two-step nature of the DOP25 and T-PCR procedures allowed labelling by incorporation of fluorescent nucleotides during the second amplification reaction (as described above). Amplified cells with a known chromosomal abnormality

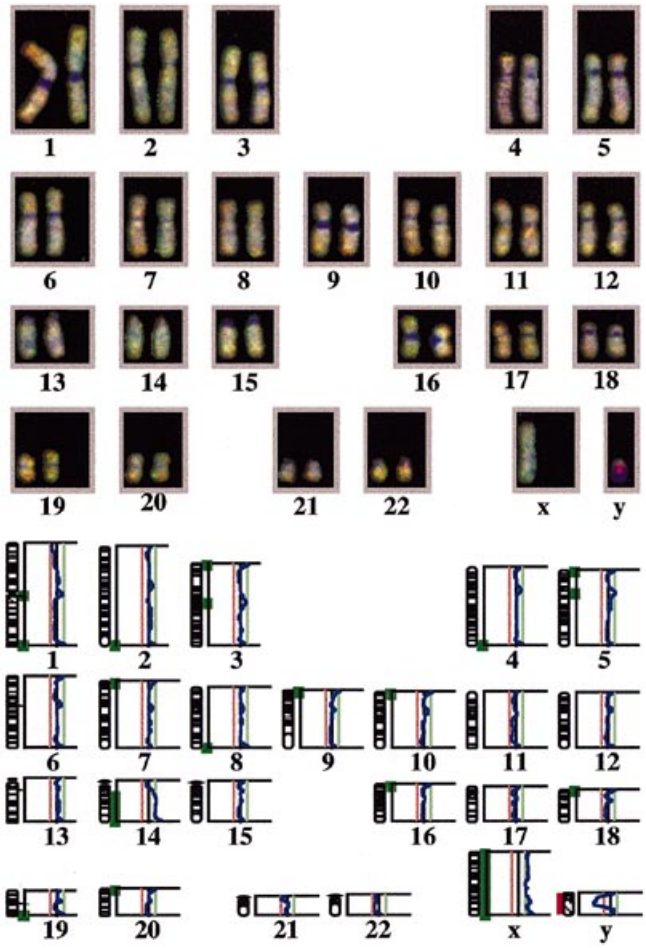


**Figure 1.** DOP-PCR amplified single cells used for CGH. The DOP-PCR product from a single buccal cell (46XY) was labelled red while the product of a single fibroblast (47,XX,+14) was labelled green. Ratios are: 1.2:1 (red line); 0.8:1 (green line). The blue line depicts the mean ratio determined by analysis of five metaphase spreads. The excess of green fluorescence on chromosome 14 confirms trisomy 14 in the fibroblast cell.

were coded for use in a blind study. The WGA products generated from the chromosomally normal and the aneuploid single cells, labelled with different colours, were precipitated together in the presence of 30 μg of Cot-1 DNA and 10 μg of salmon sperm DNA. The resultant DNA pellet was dried and resuspended in 10 μl of hybridisation mixture (50% deionised formamide, 10% w/v dextran sulphate, 2× SSC, 0.1 mM EDTA, pH 8, 0.2 mM Tris–HCl, pH 7.6). Prior to hybridisation, probes were denatured at 75°C for 10 min and allowed to preanneal at 37°C for 30 min.

Normal metaphase spreads were prepared according to standard protocols and the slides aged for ~3 days at room temperature. Slides were washed in PBS for 5 min at room temperature, dehydrated through an alcohol series (70, 90, 100% ethanol for 5 min each) and air dried. The slides were treated with 100 μg/ml RNase A in 2× SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7) and incubated for 1 h at 37°C in a humidified chamber. The RNase was removed with two 5-min washes in 2× SSC at room temperature. Slides were washed in proteinase K buffer [2 mM calcium chloride ($CaCl_2$), 20 mM Tris–HCl, pH 7.5] at 37°C for 5 min, before a 7-min treatment with proteinase K (50 ng/ml in

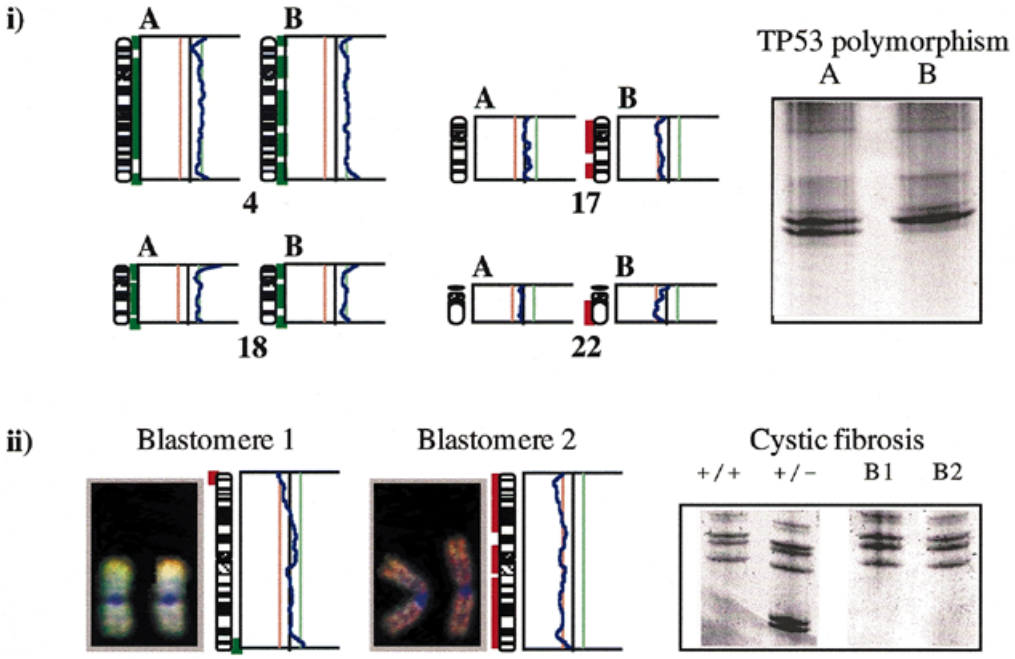


**Figure 2. (i)** Analysis of DOP-PCR product generated from two samples (A and B) microdissected from histologically distinct regions of the same ovarian tumour. The only chromosomes to display abnormal CGH profiles are those shown (green, tumour DNA; red, 46,XY DNA). Loss of chromosome 17 in sample B but not in A was indicated by CGH and by loss of heterozygosity (SSCP) for a polymorphism in exon 4 of the TP53 gene (17p13). **(ii)** Investigation of DOP-PCR amplifications of single cells biopsied from a four-cell human preimplantation embryo. The embryo was found to be mosaic: one cell had a balanced chromosome complement (blastomere 1) apparently 46,XX; three cells had a deficiency of chromosome 1 material (e.g. blastomere 2) and were probably 45,XX, –1. None of the cells carried the cystic fibrosis causing ΔF508 mutation as revealed by SSCP.

proteinase K buffer) at 37°C. Following a brief immersion in PBS:1% $MgCl_2$ the slides were fixed with paraformaldehyde (1% paraformaldehyde, 1% w/v $MgCl_2$ in PBS) for 10 min at room temperature. After this step the slides were washed in PBS, passed through an alcohol series and left to air dry. The RNase, proteinase and paraformaldehyde steps of the protocol could be omitted if the slides were free of cell debris and cytoplasm.

Slides were denatured in 70% deionised formamide/2× SSC at 75°C for 5 min before being plunged into ice-cold 70% ethanol, dehydrated through an alcohol series and air dried. Finally the probe, prepared and denatured as previously described, was added to the slides under a coverslip, sealed with rubber cement and left to hybridise at 37°C in a humidified chamber for 72 h.

Post-hybridisation washes were carried out at 45°C; 3 × 10 min 50% formamide/2× SSC followed by 3 × 10 min 2× SSC. The slides were then placed in 4× SSC/0.1% Tween 20 detergent for 10 min before being washed in distilled water for a further 10 min. The slides were dehydrated through an ethanol series, air dried and mounted in anti-fade medium (Vector Labs) containing diamidophenylindole (DAPI). Image capture was carried out using a Zeiss Axioskop microscope, Photometrics KAF1400 CCD camera, and SmartCapture software supplied by Vysis. Image analysis was performed using Vysis Quips CGH software.

DOP-PCR methods provided very good CGH results and in a blind study of single amniocytes allowed reliable detection of trisomies 13, 14 (Fig. 1), 18 and 21, as well as determination of sex. The hybridisation of DOP-PCR products to normal metaphase chromosomes produced strong even signals with no obvious sites of amplification deficiency or excess. DOP25 was particularly successful as incorporation of fluorescent nucleotides during the amplification reaction produced significantly brighter signals than achieved using nick translation and allowed more sensitive determination of imbalances affecting the smaller chromosomes. The increased intensity also suggested that it might be possible to reduce the length of the hybridisation and still obtain sufficient signal strength for single cell CGH. Preimplantation genetic diagnosis protocols generally require tests that can be completed within 48 h. Analysis after a 24 h hybridisation successfully identified sex and trisomy for chromosomes 13, 14 and 18; however, detection of trisomy 21 was not accomplished consistently.

T-PCR also gave accurate CGH results, but demonstrated a disproportionately high level of hybridisation to centromeric and telomeric regions and reduced signal intensity elsewhere on the chromosomes. The apparent preferential amplification of repetitive DNA sequences localised to these regions necessitated an increase in the amount of Cot-1 DNA used for competitive *in situ* suppression, and consequently increased the cost of the procedure. Single cell alu PCR also had some success when combined with CGH, and allowed efficient sexing. However, the hybridisation was uneven, showing an extreme bias towards regions of the genome rich in alu repeats with low CGH sensitivity in the alu-deficient areas. Attempts to perform CGH with DNA amplified using the PEP technique failed to give any accurate results as signal intensity was minimal.

The success of DOP-PCR for both molecular and cytogenetic analyses has allowed us to conduct 90 separate PCR amplifications and also CGH on the same cell. The analysis of large numbers of specific loci combined with the detection of aneuploidy in single cells may have a profound impact on a number of research areas. We have applied our DOP-PCR and CGH protocols to microdissected

ovarian tumour samples each consisting of 10–50 cells. In one case CGH revealed deletion of chromosome 17 in cells from one region of a tumour, but not in cells sampled from a second histologically distinct area <5 mm distant. Molecular study of the DOP-PCR product from the first sample revealed loss of heterozygosity involving the TP53 gene, which maps to chromosome 17p, thus complementing the CGH data (Fig. 2). Such experiments may help us to correlate the affect of specific genetic changes on tumour microhistology, and deduce the nature and order of alterations associated with tumour progression.

A further exciting application of single cell CGH is in developmental genetics where it will shed light on the mechanisms underlying the unprecedented levels of chromosomal mosaicism reported in human embryos at the cleavage stage (16), a factor which may influence the relatively low efficiency of human *in vitro* fertilisation techniques. CGH analysis of each cell from disaggregated human preimplantation embryos has revealed normal embryos, mosaic embryos containing two cell lines (Fig. 2), embryos with differing chromosomal losses and gains in each cell (chaotic chromosome segregation), and some cases of chromosome breakage leading to complete or partial loss of chromosome arms.

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