

Carrier-specific breakpoint-spanning DNA probes: an approach to preimplantation genetic diagnosis in interphase cells

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Carriers of chromosomal inversions or other balanced rearrangements represent a significant fraction of patients in in-vitro fertilization (IVF) programmes due to recurrent reproductive problems. In most cases, chromosomal imbalance in fertilized oocytes is incompatible with embryo survival leading to increased rates of spontaneous abortions. Assuming that a fraction of the germ cells is karyotypically normal, these patients would greatly benefit from efficient procedures for generation and use of breakpoint-specific DNA hybridization probes in preconception and preimplantation genetic diagnosis (PGD). We describe the generation of such patient-specific probes to discriminate between normal and aberrant chromosomes in interphase cells. First, a large insert DNA library was screened for probes that bind adjacent to the chromosomal breakpoints or span them. Then, probe and hybridization parameters were optimized using white blood cells from the carrier to increase in hybridization signal intensity and contrast. Finally, the probes were tested on target cells (typically polar bodies or blastomeres) and a decision about the colour labelling scheme was made, before the probes can be used for preconception or preimplantation genetic analysis. Thus, it was demonstrated that cells with known structural abnormalities could be detected, based on hybridization of breakpoint spanning yeast artificial chromosome (YAC) DNA probes in interphase cells.

Key words: aneuploidy/chromosome rearrangements/FISH/in-vitro fertilization/PGD

Introduction

Carriers of structural chromosomal rearrangements such as pericentric inversions or reciprocal translocations have an increased risk of spontaneous abortions (Haagerup and Hertz, 1992) and of producing offspring with genetic abnormalities (Kleczkowska *et al.*, 1987; Ayukawa *et al.*, 1994). Familial pericentric inversions have also been associated with trisomy 9, 11, 18, 21, and 22, double aneuploidy, and sex chromosome

aneuploidy (Kaiser 1984; Kleczkowska *et al.*, 1987). These events are consequences of unbalanced gametes that arise from crossing-over within the inverted segments in inversion heterozygotes. Madan (1995) proposed a mechanism for the formation of unbalanced gametes and suggested that balanced and unbalanced gametes occur in equal numbers. Chromosome analyses of spermatozoa from pericentric inversion heterozygotes, however, found 31% unbalanced gametes in a carrier of an *inv(3)* (Martin, 1991) and 25% in an *inv(7)* carrier (Navarro *et al.*, 1993) suggesting the existence of mechanisms which impair survival of unbalanced gametes. Gardner and Sutherland (1989) estimated the overall risk of a pericentric inversion carrier producing offspring with an unbalanced karyotype at 1–10%.

Abnormal offspring and spontaneous abortions can be alleviated by performing preimplantation genetic diagnosis (PGD) before embryo transfer. Healthy normal babies have been born after PGD of X-linked genetic diseases, single gene defects, and aneuploidy (Verlinsky *et al.*, 1994). Due to technical problems related to the complex organization of interphase chromatin and lack of appropriate detection tools, PGD of structural abnormalities has rarely been attempted and, so far, has been demonstrated only for Robertsonian translocations (Munné *et al.*, 1996a). Fluorescence in-situ hybridization (FISH) is the method of choice for analysis of interphase cells, and applications of FISH for enumeration of chromosomes in sperm nuclei and interphase cells from preimplantation embryos have been described recently (Delhanty *et al.*, 1993; Munné *et al.*, 1994a; Harper *et al.*, 1995; Rousseaux *et al.*, 1995a, b; Dyban *et al.*, 1996; Rademaker *et al.*, 1997; Staessen and Van Steirteghem, 1997; Guttenbach *et al.*, 1997; Robbins *et al.*, 1997). For the detection of structural abnormalities, however, specific probes either flanking or spanning the breakpoints (Tkachuk *et al.*, 1991) must be developed for each case.

The existence of well characterized genomic libraries comprised of recombinant clones with large insert DNA fragments now allows rapid and thus cost-efficient isolation of breakpoint-spanning probes. In the study presented here, we used clones from the CEPH/Genethon yeast artificial chromosome (YAC) library (Weissenbach *et al.*, 1992) to prepare probes that span breakpoints on the short and long arms, respectively, of chromosome 6 in cells from a carrier of an *inv(6)*. Our patient, however, became pregnant during the course of probe development, and the application of probes described in this study shifted from first polar body analysis for structural aberrations (Munné *et al.*, 1996a) to detection of numerical aberrations involving either one or both arms of chromosome 6.

This study describes a first successful attempt to develop

probes for PGD of a chromosomal inversion and lays the groundwork for routine production of patient-specific probes. Using appropriate carrier-specific probes, the same methodology will allow detection of a variety of structural chromosome abnormalities ranging from intrachromosomal rearrangements to microdeletions, thus enabling PGD in a large group of IVF patients.

Materials and methods

Preparation of metaphase chromosome spreads from patient cells

Patient A.K., a 27 year old female Caucasian, was referred to the Saint Barnabas Medical Center due to her history of reproductive failures. Routine medical cytogenetic analysis by G-banding was performed at a hospital in the patient's home town and demonstrated a karyotype 46, XX, inv(6)(p23q23.1). Peripheral blood was obtained from the patient by venipuncture and mailed to us.

Metaphase spreads were made from short-term cultures of the patient's lymphocytes grown for 72 h in RPMI 1640 supplemented with 20% fetal calf serum, 2% penicillin and 4% phytohaemagglutinin [Life Technologies Inc. (LTI), Gaithersburg, MD, USA] according to the procedure described by Harper *et al.* (1981). Cultures were blocked for 17 h with methotrexate (10^{-5} M, Sigma, St Louis, MO, USA), followed by incubation in RPMI containing thymidine (10^{-5} M, LTI) for 5 h. Cells were blocked in mitosis during a 10 min treatment with colcemid (0.12 μ g/ml, LTI), harvested and incubated in 75 mM KCl for 15 min at 37°C. The cells were then spun down, and approximately 10^7 cells were fixed in 5 ml freshly made acetic acid/methanol (1:3 v/v). The fixative was changed twice and cells were dropped on ethanol cleaned slides. Slides were stored for at least 2 weeks in ambient air at room temperature, then placed under nitrogen in sealed plastic bags at -20°C until used.

Selection of YAC clones

Before being sent to us, patient cells are typically analysed by G-banding of metaphase cells suggesting an approximate location of the translocation breakpoints. This information is available to us prior to probe isolation. Alternatively, cytogenetic data regarding the approximate location of the breakpoints can be generated rapidly by chromosome painting. Breakpoint cloning in YAC then requires refinement of the breakpoint containing genomic interval, i.e., the region of the chromosome that has been interrupted by translocation or inversion, and identification of clones that are immediately adjacent to or span the breakpoints.

Our general scheme for selection and optimization of probes for PGD is shown in Figure 1. Using information in publicly available databases, we then select a number of clones that map to this interval as well as adjacent chromosome bands, extract and label DNA and perform the FISH analysis. Besides probes which fail to give a signal or hybridize to the wrong chromosome, 70–80% of our YAC probes typically map to the target chromosome allowing us to rapidly narrow the breakpoint region. If none of the probes spans the breakpoint, i.e. the hybridization signal is not split into two parts appearing on both derivative chromosomes, probes are recorded as mapping either distal or proximal to the breakpoint. It is important to note that this is a rather straightforward task, since each complete metaphase cell contains one normal homologue as well as one copy of each derivative chromosome. Probe positions relative to the breakpoint are then compared to the positions of clones on maps in the database. Next, we select additional probes spaced more or less evenly in the minimal breakpoint interval defined by proximal and distal probes.

This alignment of our cytogenetic map with the genetic and/or

physical map provided by the database is not a trivial task, because our cytogenetic mapping does not allow us to determine the relative order of probes; nor is the database free of errors, as shown below in our example for the short arm of chromosome 6. In most cases, however, each set of hybridizations allows us significantly to narrow the breakpoint containing interval until one or several of the probes hit or span the breakpoint.

Chromosomal breakpoints in patient A.K. had been mapped by G-banding to chromosome 6 bands p23 and q23.1. We selected YAC clones based on STS markers that map approximately in these regions and information provided in the Whitehead Institute for Biomedical Research/MIT Center for Genome Research database (<http://www-genome.wi.mit.edu/>)(Hudson *et al.*, 1995). We preferentially selected YAC clones larger than 1000 kb which appeared non-chimeric based on sequence tagged sites (STS) content mapping data. For the initial round of probe preparation, we chose clones in about 10–15 Mbp intervals to determine the interval containing the inversion breakpoints. In the second round of probe preparation, we chose clones that mapped in approximately 2 Mbp intervals and ordered them proximal or distal to the breakpoints. We continued to select and map YAC in smaller intervals until we identified clones that either spanned a breakpoint or bound closely adjacent to it.

Preparation of DNA probes

For initial mapping of YAC clones, DNA was isolated from yeast cell colonies using a standard DNA miniprep procedure (Sherman *et al.*, 1986; Weier *et al.*, 1995b). Once clones were identified that spanned or bound adjacent to the breakpoint, probe optimization was carried out by separating the YAC from the yeast DNA using pulsed field gel electrophoresis (PFGE) on a CHEF Mapper II PFGE instrument (Bio-Rad Laboratories, Richmond, CA, USA). Agarose plugs of YAC clones were prepared as follows: YAC clones from the Genethon/CEPH library (Weissenbach *et al.*, 1992) were streaked on adenine hemisulphate and casein hydrolysate (AHC) plates (Sherman *et al.*, 1986) and incubated 2–3 days at 30°C. Five to ten pink colonies were picked and cultured individually in 5 ml of AHC (-ura/-tryp) media for 48 h. Cells were allowed to settle and transferred to a 1.5 ml microcentrifuge tube and spun at 1750 g for 6 min. The supernatant was discarded and pellets were resuspended in 0.5 ml of 125 mM EDTA, pH 7.8. The tubes were then centrifuged for 10 s and the supernatant removed. Pellets were resuspended in SCE (1 M sorbitol, 0.1 M sodium citrate, 10 mM EDTA, pH 7.8) (250 μ l SCE/35 μ l of pellet) and vortexed. Then, 1.5% LMP agarose (LTI) prewarmed to 45°C was added (250 μ l agarose/35 μ l of pellet) and quickly mixed with the cells by pipetting up and down. The tubes were vortexed 1–2 s, the mix was dispensed into plug moulds (Bio-Rad) and refrigerated for 10 min. Plugs were then removed from the moulds and placed in 2 ml SCE with 100 μ l of Zymolase (INC Biomedicals Inc., Aurora, Ohio, USA) (10 mg/ml in 50 mM KPO₄, pH 7.8, 50% glycerol) and shaken at 150 r.p.m. at 30°C for time periods ranging from 2.5 h to overnight. The SCE was then removed and 2 ml of ES solution (0.5 M EDTA, pH 8.0, 1% Sarcosyl; Sigma) containing 100 μ l of Proteinase K (20 mg/ml in 10 mM Tris-HCl, pH 7.5; Boehringer Mannheim) were added and the tubes were shaken for 5–14 h. The ES solution was removed and plugs were rinsed five times in 6 ml of TE50 (10 mM Tris-HCl, pH 7.5, 50 mM EDTA, pH 7.8) for 30 min each, before being stored in TE50 at 4°C. The plugs were loaded on a 1.0% LMP agarose gel and run under the following conditions: 0.5 \times TBE (445 mM Tris, 440 mM boric acid, 10 mM EDTA) at 14°C and 6 V/cm for 43 h with an initial switch time of 75 s and a final switch time of 94 s. The gel was then stained for 30 min in 500 ml water containing 0.5 μ g/ml ethidium bromide (LTI) and rinsed twice for 30 min in 500 ml distilled water.

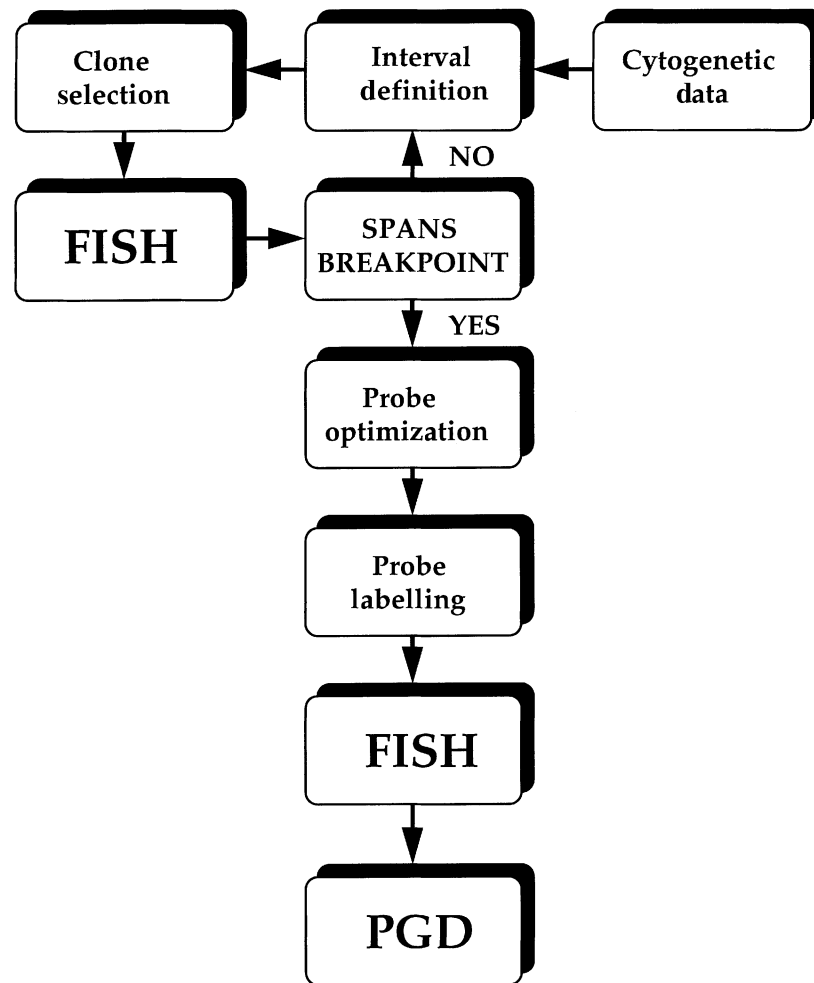


Figure 1. Schematic diagram of the steps involved in preparation of breakpoint-specific probes for use in pre-conception and pre-implantation genetic diagnosis (PGD). FISH = fluorescence in-situ hybridization.

The slice containing the YAC was then identified under brief UV illumination, excised from the gel, equilibrated in agarase buffer and digested with agarase according to the supplier's instructions (New England Biolabs, Beverly, MA, USA).

We prepared probe DNA from PFGE purified DNA of YAC clones by a 40 cycle DOP-PCR (Telenius *et al.*, 1992) using primer JUN1 (5'-CCCAAGCTTGCATGCGAATTCNNNNCAGG-3', $n = \text{ACGT}$) (Weier *et al.*, 1993) with an initial 8 cycles of primer annealing at 37°C and extension by T7 DNA polymerase (Kroisel *et al.*, 1994). This oligonucleotide primed amplification from genomic templates only when annealed at low temperature (30–40°C). The PCR products were further amplified at higher annealing temperature (50–55°C) using primer JUN15 (5'-CCCAAGCTTGCATGCGAATTC-3') and Taq polymerase for 32 cycles (Kroisel *et al.*, 1994).

The PCR products were purified by a chloroform extraction, precipitated in ethanol and resuspended in 40 µl of TE buffer, pH 7.2. DNA concentrations were measured using a TKO 100 Mini-Fluorometer (Hoeffer Scientific Instruments, San Francisco, CA). We labelled 400 ng aliquots of the DNA with either biotin or digoxigenin using the BioPrime DNA labelling system (LTI) as described (Weier *et al.*, 1994, 1995a, b).

In-situ hybridization

One microlitre of each probe along with 1 µl of human Cot1™ DNA (1 mg/ml, LTI) and 1 µl of salmon sperm DNA (20 mg/ml; 3'-5';

Boulder, CO, USA) was added to 7 µl of the hybridization master mix [78.6% formamide, 14.3% dextran sulphate in 2.9× SSC, pH 7.0 (2× SSC is 300 mM NaCl, 30 mM Na citrate)]. We denatured the hybridization mix at 76°C for 7 min and allowed it to pre-anneal at 37°C for 60 min. The slides were denatured 3.5 min at 76°C in 70% formamide/2× SSC, pH 7.0, dehydrated by immersion in 70, 85 and 100% ethanol (2 min each), and allowed to dry. We applied the hybridization mix to the slides and covered it with 22 mm×22 mm coverslips which were then sealed with rubber cement. The hybridization was allowed to proceed overnight in a moisture chamber, after which the rubber cement was carefully removed leaving the coverslip in place. The slides were immersed in 50% formamide, 2× SSC, pH 7.0 at 43°C for approximately 5 min at which time the coverslips had fallen off or became loose enough to slide off. Once the coverslips were removed, the slides were washed twice in 50% formamide, 2× SSC at 43°C for 10 min followed by two washes in 2× SSC at room temperature. Slides were then incubated in PNM [5% nonfat dry milk (Carnation®), 1% Na azide, in PN buffer (0.1 M sodium phosphate buffer, pH 8.0, 1% Nonidet-P40)] at room temperature for 5 min. Fifty microlitres of fluorescein avidin DCS (avidin-FITC, 2.5 µg/ml in PNM; Vector Labs, Burlingame, CA, USA) and 50 µl of anti-digoxigenin-rhodamine (anti-dig.-rhodamine, 2.0 µg/ml in PNM; Boehringer Mannheim) were mixed and applied to the slides. The slides were covered with plastic coverslips and incubated 20 min in a moisture chamber at 20°C. The slides were

Table I. Yeast artificial chromosome (YAC) clones for the breakpoint on chromosome 6p23

Cycle number	Clone	Position (cM)	Position (cR)	YAC size (kb)	FISH result	Selected STS
1	800a6	13, 18	60, 61	1650	wrong chromosome	D6S296 D6S470
	871e7	27	63	1180	no signal	D6S429
	938e7		69	700, 1000	distal	FB15B5
	801c11		90	1580	proximal	AFMB277ZD5 D6S1453
2	747f10	33	51, 64	400, 800	distal	D6S274
	857b11		51	820	distal	AFM189YE3 WI3897
	771h1			1780	distal	WI3897
	850f5			900	distal	WI3897
	755d2		79, 71 585	1150	two signals	AFM161YF4 D6S1056
3	917a1		79	1350	distal	AFM161YF4
	810d1		79	320, 400	distal	AFM161YF4
	814b9	35	75	1400	distal	D6S285
	805e5	35	75	1500	distal	D6S285
4	966e10		86, 87 91	870	distal	WI5592 WI5267
	874b3	45	96	1430	spanning	D6S276 WI3998
	759h10	42, 44	96	450	spanning	D6S299 WI3998
	901a10		99	1130	proximal	GATAP19326
	967h3	46		1380	wrong chromosome	D6S464 WI5500
5	949f8			1590	distal	WI4818
	935a8	42	96	1160	spanning	D6S461 WI3998
	872h10	44		1630	no signal	D6S299

STS = sequence tagged sites.

FISH = fluorescence in-situ hybridization.

then washed three times for 10 min each in $2\times$ SSC. Excess liquid was drained from the slide and $7\ \mu\text{l}$ 4,6-diamino-2-phenylindole (DAPI) (0.5 $\mu\text{g}/\text{ml}$; Calbiochem, La Jolla, CA, USA) in antifade solution [0.1% p-phenylenediamine dihydrochloride (Sigma), $0.1\times$ PBS (LTI), 45 mM NaHCO_3 , 82% glycerol, pH 8.0] were applied and covered with a $22\ \text{mm}\times 22\ \text{mm}$ glass coverslip. Fluorescence microscopy was performed using a Zeiss Axioskop[®] microscope with a filter set for simultaneous observation of Texas Red and FITC (ChromaTechnology, Brattleboro, VT, USA) and a separate filter for DAPI detection. Images were collected using a cooled CCD camera (Photometrics, Tucson, AZ, USA) and a Sun Spark[®] station (Sun Microsystems, Pleasanton, CA, USA). Further processing and printing of the images was done using Photoshop V2.5.1 (Adobe Software, Mountain View, CA, USA) for the Power Macintosh[®].

Preparation of blastomeres from normal embryos donated for research

Embryos for this study were donated for research by patients enrolled in the IVF programme of The Institute for Reproductive Medicine and Science of Saint Barnabas Medical Center and, in accordance with guidelines set by the internal review board of Saint Barnabas Medical Center, written consent was obtained from the patients in each case. Only non-arrested, monospermic embryos developing from bipronucleated zygotes were used for this study.

Embryos were biopsied or disaggregated on day 4 of development as described (Grifo *et al.*, 1992) and cells were fixed following our protocol for blastomeres (Munné *et al.*, 1994a, 1996b). Briefly, a hole was drilled through the zona pellucida with acidified Tyrode's solution (pH = 2.4) (Gordon *et al.*, 1986) and several blastomeres were removed from each embryo by micromanipulation (Grifo *et al.*, 1992).

Each biopsy took approximately 10 min per embryo. Blastomeres were fixed individually on glass slides using a slight modification of Tarkowski's technique (Tarkowski, 1966; Munné *et al.*, 1993). Each blastomere was placed in a culture dish containing hypotonic solution (1% sodium citrate in water, 6 mg/ml bovine serum albumin, Sigma) for five min at 25°C , then transferred into a small volume of hypotonic solution on a slide. Ten microlitres of acetic acid:methanol fixative (1:3 v/v) were dropped on top, while observing the cell under a Wild M3Z[®] stereo microscope (Leica, Deerfield, IL, USA). The fixative was spread by continuous and gentle blowing until the cytoplasm dissolved. The position of the nucleus was marked with a tungsten-carbide tip pencil. The fixation process lasted less than 10 min/blastomere.

The probes used for blastomere analysis were biotin- and digoxigenin-labelled (6p and 6q, respectively). A $1\ \mu\text{l}$ aliquot of each probe, $1\ \mu\text{l}$ of Cot1[™] DNA, $1\ \mu\text{l}$ of salmon sperm DNA and $7\ \mu\text{l}$ of hybridization master mix were combined, applied to the glass slide containing fixed blastomeres and covered with an $18\ \text{mm}\times 18\ \text{mm}$ coverslip. The slide was then placed for 2 min on a slide warmer preheated to 78°C , sealed with rubber cement, and placed in a moisture chamber at 37°C overnight. Following the hybridization, the slides were washed at 72°C in $1\times$ SSC for 2 min, and bound probes were detected with avidin-FITC and anti-dig.-rhodamine as described previously (Weier *et al.*, 1994, 1995b). The slides were then mounted with $10\ \mu\text{l}$ of DAPI counterstain in antifade solution and observed with a fluorescent microscope (BX60, Olympus, Melville, NY, USA) equipped with a triple-band pass filter set (ChromaTechnology). A scoring criterion for differentiating false-positives and false-negatives from mosaicism has been previously described (Munné *et al.*, 1994a).

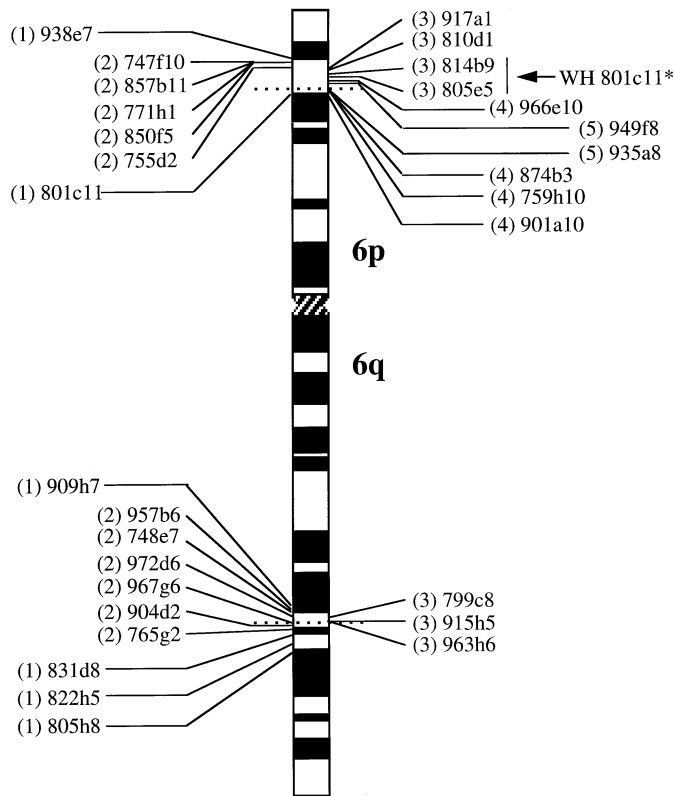


Figure 2. Relative map positions of yeast artificial chromosome (YAC) clones along chromosome 6. Breakpoint regions are indicated by dotted horizontal lines. Numbers preceding clone names indicate the cycle in which the clone was selected. We performed five and three cycles of clone selection for the breakpoints on the short and long arm of chromosome 6, respectively. The approximate location of clone 801c11 as suggested by data from the Whitehead/CEPH database is indicated by a vertical line and an asterisk following the YAC name.

Results

Clone selection and probe mapping

Chromosome 6p

All clones selected are compiled in Table I. The STS content data and clone positions on the genetic and radiation hybrid maps (measured in cM and cR, respectively) were provided by the Whitehead Institute. It is important to note that the Whitehead YAC mapping database was updated while our study was in progress. For example, the database used for clone selection lacked much of the radiation hybrid mapping data, which provide a more linear scale for physical mapping than the recombination frequencies used to construct the genetic maps. In the first round of probe preparation, the following YAC clones were selected for breakpoint mapping on the short arm of chromosome 6: 800a6, 871e7, 938e7, and 801c11 (Figure 2). Clones 938e7 and 801c11 mapped distal and proximal, respectively, to the breakpoint, while 871e7 gave no signal and 800a6 mapped to the wrong chromosome. Thus, clone 801c11 mapped to a location proximal to that indicated in the database (Figure 2). Because the database indicated clone 871e7 to map outside of the interval defined by clones 938e7 and 801c11, we did not make further attempts to map this clone. The following YAC mapping in the interval

defined by 938e7 and 801c11 were identified in the database and selected: 747f10, 857b11, 771h1, 850f5, and 755d2. All of the second round probes were found to map distal to the breakpoint (Figure 2) and 755d2 gave two signals on chromosome 6. In the third round, we selected YAC clones 917a1, 810d1, 814b9, 805e5, 926g2, and 798f7. Of these, 917a1, 810d1, 814b9, and 805e5 mapped distal to the breakpoint (Figure 2). Clones 926g2 and 798f7 did not produce pink colonies, and were not further processed.

For the fourth round of probe selection, we chose and mapped seven YAC clones for which the database indicated a more proximal location. Hybridization indicated that clones 874b3 and 759h10 spanned the breakpoint, 966e10 mapped distal to the breakpoint and 901a10 mapped proximal to the breakpoint (Figure 2, Figure 3A, B). The clone 967h3 mapped to the wrong chromosome, and 825c1 failed to show pink colonies. Once we had probes that spanned the breakpoint, we selected YAC 949f8, 935a8, and 872h10 to map more accurately the breakpoint and to provide a larger set of probes from which to choose. Of these, 935a8 spanned the breakpoint, 949f8 mapped distal to the breakpoint (Figure 2), and 872h10 did not give a hybridization signal.

Chromosome 6q

The clones chosen for delineation of the breakpoint on the long arm of chromosome 6 are listed in Table II. In the first round of probe preparation, the following probes were selected: 909h7, 831d8, 822h5, 874g2, and 805h8. The clones 831d8, 822h5, and 805h8 mapped distal to the breakpoint, while 909h7 was found proximal to the breakpoint and 874g2 did not give a hybridization signal (Figure 2). Based on these results, another six clones were selected: 967g6 spanned the breakpoint, 957b6, 748e7, and 972d6 mapped proximal, while 904d2 and 765g2 mapped distal to the breakpoint (Figure 2). Once we had identified a clone that spanned the breakpoint (i.e., clone 967g6), we selected the partially overlapping YAC clones 954e4, 799c8, 915h5, and 963h6. Clones 915h5 and 963h6 both spanned the breakpoint, while clone 799c8 mapped proximal to the breakpoint and gave a second signal on a different chromosome (Figure 2), and clone 954e4 mapped entirely to a different chromosome.

In summary, of 39 clones selected, three mapped to chromosomes other than chromosome 6, three produced no hybridization signal and four produced only white colonies assumed to be wild-type yeast. However, we identified a total of six clones that spanned either breakpoint (three clones on 6p and 6q, respectively).

Figure 3C–E shows representative results of breakpoint spanning probes hybridized to the normal and derivative chromosomes 6 and interphase cells respectively. In the hybridization shown in Figure 3C–E, we combined a probe prepared from clone 963h6 (6q, red) with a probe made from 874b3 (6p, green).

Blastomere analysis

The intensity of signals obtained after hybridization of fluorochrome-labelled probes to blastomeres was rated on a scale ranging from 'three' (bright) to 'zero' (non-visible). According to this scale, we rated the probe made from clone

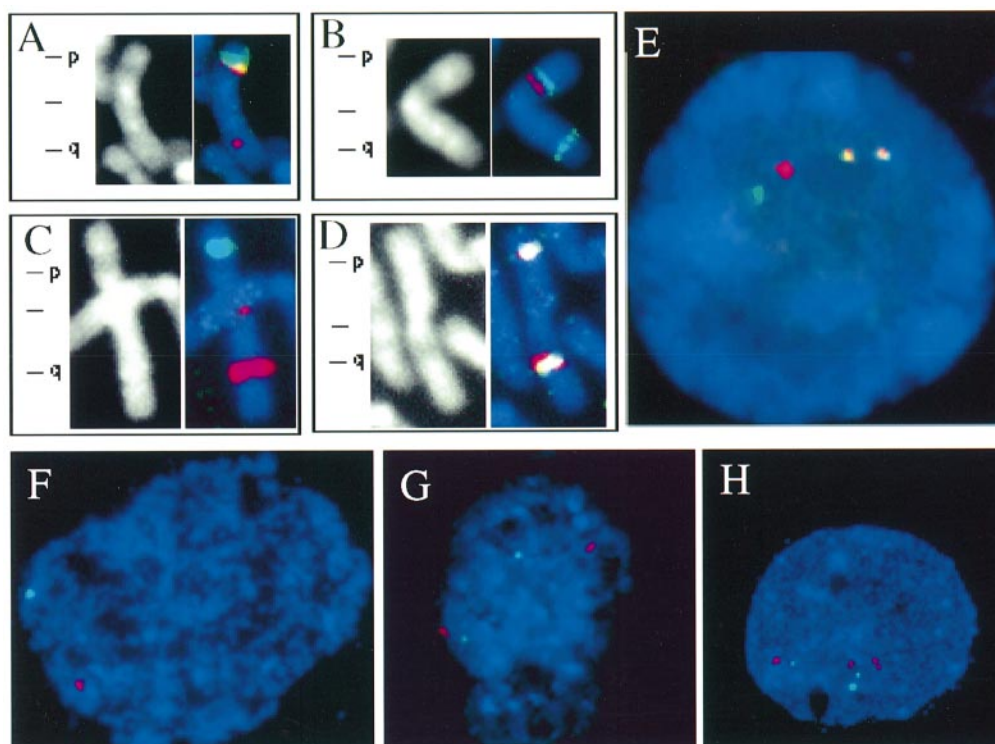


Figure 3. Pattern observed after hybridization of fluorochrome labelled DNA probes to metaphase chromosomes from patient lymphocytes and blastomeres donated for research purposes. (A–D) Hybridization of yeast artificial chromosome (YAC) DNA probes to metaphase spreads prepared from white blood cells of a patient with *inv(6)*. These images show only partial metaphases containing either a normal (A, C) or derivative chromosome 6 (B, D). To the left, short and long arms of the chromosomes are indicated by p and q, respectively. Images displaying the DAPI staining alone and hybridization results are shown in the centre and to the right, respectively. (A) Normal chromosome 6 and (B) derivative chromosome 6 (*inv(6)*) hybridized with a probe spanning the breakpoint on 6p (874b3, green) and a proximal probe (901a10, red). On the *der(6)* (B), approximately half the breakpoint spanning probe has been translocated to the long arm, while the red signal can be observed on the short arm. (C) Normal chromosome 6 and (D) derivative chromosome 6 (*inv(6)*) hybridized with breakpoint spanning probes prepared from clones 963h6 (long arm, red) and 874b3 (short arm, green). On the *der(6)*, approximately half of each breakpoint spanning probe has been translocated to the opposite arm resulting in a yellow signal (overlap of red and green signal domains) on each arm. (E) An interphase cell (lymphocyte) from the patient hybridized with the breakpoint spanning probe set. The normal homologue of chromosome 6 gives one red and one green signal domain. The rearrangement on the *der(6)* results in two yellow signals. (F–H) Analysis of human blastomeres. (F) A blastomere from embryo H hybridized with the chromosome 6 probes showing one red and one green signal (haploid or monosomic phenotype in a mosaic embryo). (G) A blastomere hybridized with the final breakpoint spanning probes showing three red and three green signals (disomic phenotype). (H) A blastomere hybridized with the breakpoint spanning probes showing three red and three green signals (trisomic phenotype).

963h6 (6q, red) a three and assigned a one to the probe made from clone 874b3 (6p, green). In addition, the probe for the breakpoint on chromosome 6p appeared to cross-hybridize weakly with another set of chromosomes.

We analysed 75 blastomeres from 10 embryos by simultaneous hybridization of probes from clones 874b3 (6p, green) and 963h6 (6q, red). The results are summarized in Table III. Of 75 blastomeres analysed, 71 showed the same number of 6p and 6q signals per cell. In the remaining four cells, the signals for the 6p probe were not clear enough to make a diagnosis.

Among the 10 embryos analysed, six were normal (embryos A, B, F, G, I, J), three (embryos D, E, H) were mosaics, and one was haploid (embryo C) (Table III). The haploid embryo was diagnosed as such after the re-hybridization with X and Y probes following a previously published re-hybridization protocol (Benadiva *et al.*, 1996). Each nucleus of embryo C showed a single X probe signal in the absence of a Y-specific signal. Embryos E and D were classified as mosaics, being

probably diploid with haploid or polyploid cells. Analysing only a single chromosome type, it was not possible to determine whether a cell was monosomic or haploid, nor to differentiate between trisomy or triploidy. Nevertheless, if these two embryos had mitotic non-disjunction, the number of signals in different cells should have been compensated, which did not occur in these cells. In addition, it was unlikely that they were FISH errors, because the same number of signals for the two probes were found in each cell. Therefore they were probably $2n/3n$ (embryo D) and $2n/n/4n$ (embryo E) mosaics. These types of mosaics are common in human embryos (Munné *et al.*, 1994b). For the same reasons, embryo H could have been either a $n/3n$ mosaic or a monosomic/trisomic mosaic.

Typical hybridization results obtained with normal and abnormal embryos are displayed in Figure 3F–H. This figure shows the results using cells monosomic for chromosome 6 (Figure 3F) and normal disomic cells (Figure 3G) showing one each or two each red and green signal domains, respectively. We analysed five cells from embryo H all of which showed three

Table II. Yeast artificial chromosome (YAC) clones for the breakpoint on chromosome 6♀23.1

Cycle number	Clone	Position (cM)	Position (cR)	YAC size (kb)	FISH result	Selected STS
1	909h7	131, 132	686, 688	1360	proximal	D6S267 D6S412
	831d8	142	712, 719	1430	distal	WI3398 D6S262
	822h5	150	736, 739	1600	distal	D6S292 WI5077
	874g2	156	751	930	no signal	D6S453
	805h8	768	1220		distal	WI5074
2	957b6		693	1070	proximal	D6S1712 IB477
	748e7		693, 691	1090	proximal	WI8673 IB477
	972d6		699	1190	proximal	D6S1039
	967g6	138	701, 703	330	spanning	D6S407 WI9588
	904d2			1150	distal	D6S1705
	765g2		708, 706	1780	distal	D6S1040 WI3653
3	954e4			1060	wrong	D6S1702
				1080	chromosome	D1S422
	799c8	138	695	1170	two signals	D6S408 D7S635
	915h5			1600		
	963h6		701	1310	spanning	D6S1702 WI9588

STS = sequence tagged sites.

FISH = fluorescence in-situ hybridization.

signals for both chromosome arms suggesting trisomy 6 (Figure 3H). The blastomere shown in Figure 3H presented two compact red hybridization domains and one domain which appears to be split into two dots. Following our scoring criteria, split domains such as this one were scored as one domain, if the two dots were less than one domain diameter apart (Munné *et al.*, 1994a).

Discussion

We developed a simple, yet highly efficient scheme for preconception/implantation genetic diagnosis (PGD) of chromosomal inversions that can be extended to the detection of a great variety of structural chromosome changes. While several techniques are available to detect structural abnormalities in cells from cultures, most of them such as conventional karyotyping and comparative genomic hybridization (Kallioniemi *et al.*, 1992) are not suitable to produce results from a single interphase cell. Techniques based on in-vitro amplification of specific DNA targets have demonstrated value for the detection of microdeletions (Kent-First *et al.*, 1996; Qureshi *et al.*, 1996), but cannot be applied in cases of balanced translocations. Our test relies on chromosomes present in first polar bodies or blastomeres and fluorescence in-situ hybridization (FISH) using locus-specific probes for the rearranged chromosomes. The probes were prepared so that they can be used in the analysis of metaphase cells as well as interphase cell nuclei, which do not allow direct analysis of chromosomes.

Application of this test in the IVF setting increases a couple's chances of sustaining pregnancy by transferring only embryos which were either confirmed not to carry a particular chromo-

somal abnormality or found to be chromosomally balanced. While the probes reported here were specific for a pericentric inversion, the method may be used to generate probes for the detection of any chromosomal inversion, reciprocal translocation or deletion in interphase cells or polar bodies. Since the aberration can be of either maternal or paternal origin, the PGD is to be done on first polar bodies or blastomeres, respectively. The polar bodies can be used, if the breakpoints suggest that no interstitial chiasmata will be formed. Otherwise, blastomeres should be used because the polar bodies will contain a chromosome with asymmetric chromatids. Smaller IVF centres that lack the facilities or expertise to perform PGD could collect patient samples and send them to a PGD centre where probes would be developed and the analysis performed.

One practical limitation of the current method is the time frame of probe development. Once the YACs have been selected, they are retrieved from the library and grown on agar plates for 2–3 days until colonies have formed. The colonies containing the YAC are then picked and cultured for an additional 2 days. Next, the DNA is isolated and purified requiring another 2 days. The following 5–7 working days are usually occupied with probe labelling, FISH, image acquisition and analysis. Taken together, even the straightforward approach to probe isolation and labelling using a 'perfect' library and mapping database may require three rounds of clone selection and, thus, about 6 weeks to complete.

It is, however, possible to increase the number of patients for which probes are developed by working on more than one patient probe set in parallel. However, because of physical limitations and the increased time that would be required for each step, it is not practical to work with more than three

Table III. Fluorescence in-situ hybridization (FISH) results using ICSN nomenclature

Embryo	Chromosome 6 FISH results	Diagnosis
A	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 5]	normal
B	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 5]	normal
C ^a	nuc ish 6p23(D6S1033×1),6q23.1(D6S276×1), Xcen(DXZ1×1) [<i>n</i> = 6]	haploid
D	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 2] nuc ish 6p23(D6S1033×3),6q23.1(D6S276×3) [<i>n</i> = 1]	mosaic
E	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 5] nuc ish 6p23(D6S1033×1),6q23.1(D6S276×1) [<i>n</i> = 1] nuc ish 6p23(D6S1033×4),6q23.1(D6S276×4) [<i>n</i> = 1]	mosaic
F	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 8] nuc ish 6p23(D6S1033×nd),6q23.1(D6S276×2) [<i>n</i> = 1]	normal
G	nuc ish 6p23(D6S1033×2), 6q23.1(D6S276×2) [<i>n</i> = 7] nuc ish 6p23(D6S1033×nd),6q23.1(D6S276×2) [<i>n</i> = 1]	normal
H	nuc ish 6p23(D6S1033×3),6q23.1(D6S276×3) [<i>n</i> = 5] nuc ish 6p23(D6S1033×1),6q23.1(D6S276×1) [<i>n</i> = 4]	mosaic
I	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 8]	normal
J	nuc ish 6p23(D6S1033×2),6q23.1(D6S276×2) [<i>n</i> = 13] nuc ish 6p23(D6S1033×nd),6q23.1(D6S276×2) [<i>n</i> = 2]	normal

^aXY re-hybridization.*n* = number of cells.

nd = not determined.

patients at any time. A more efficient and time saving method would be to perform inter Alu-PCR on the YAC of interest (Lengauer *et al.*, 1992). A PCR DNA amplification directly from the yeast cells might allow preparation of probes in 1–2 days using YAC directly from the library or following short term culture thereby eliminating most of the delay caused by YAC culturing and DNA isolation. New, high resolution physical maps for individual chromosomes such as the chromosome 6p map published after completion of this study (Bray-Ward *et al.*, 1996), will greatly facilitate YAC walking and breakpoint cloning. Our mapping data of the breakpoint on chromosome 6p, incidentally, was found to be in agreement with this high resolution YAC map (Bray-Ward *et al.*, 1996), narrowing the breakpoint location on the short arm to the three overlapping clones 759h10, 874b3 and 935a8.

The integrity of the YAC library as well as the accuracy of the database will significantly impact on the time required to produce a final set of probes. In our example, clone 801c11 did not map to the location shown in the database (Figure 2) and we selected clones in the second round that mapped well distal of the breakpoint (Figure 2). As a result, a total of five rounds of YAC selection were required for the p arm compared to only three rounds of selection and mapping for probes for the breakpoint on the long arm of chromosome 6.

Compared to most other cytogenetic investigations, PGD requirements of hybridization efficiency are extremely high. Since only one or two cells are available for analysis, hybridization failures cannot be allowed. Probe optimization and hybridization protocol development therefore played very important roles after the initial probe isolation. This involved testing of different reporter molecule moieties and cellular targets to mimic the PGD situation.

Our patient became pregnant prior to completion of the probe development. Thus, we had no opportunity to apply the probes for analysis of her first polar bodies. However, the utility of these probes for detection of chromosomal abnormalities could be demonstrated by analysis of blastomeres

from donated embryos. The results (Table III) demonstrated an unexpected high incidence (30% considering the haploid cells were derived from an unfertilized egg) of embryos with what appeared to be numerical abnormalities involving chromosome 6. Further studies investigating the ploidy of our 10 embryos are pending. There is, however, no indication of structural changes affecting chromosome 6 in these embryos. Failure to give a clear hybridization signal was observed with the chromosome 6p breakpoint-specific probe in 5.3% of the cells (four of 75 cells). This happened in cells from embryos which presented a majority of normal cells and were therefore considered normal.

The observed high rate of mosaic embryos (three of 10 embryos) is alarming and should be considered when estimating the power of single cell analysis. Although it is yet unknown how mosaicism will affect the detection of structural abnormalities, we previously studied numerical abnormalities in arrested embryos and found very high rates of mosaic embryos (Munné *et al.*, 1993, 1994a). To estimate the mosaicism rates in normal embryo development, however, additional studies on normally developing embryos are required.

The use of two probes labelled differently for respective chromosome arms is an intriguing alternative to the use of centromeric DNA repeat probes for chromosome enumeration. The results presented here using carrier lymphocytes and donated embryos clearly demonstrate the versatility of this type of probing for simultaneous detection of structural and numerical aberrations. Furthermore, hybridizing two different probes for the chromosome arms provides a control mechanism for chromosomally normal cells. This should lead to higher confidence in FISH results and facilitate the selection of embryos for transfer after PGD.

In summary, we have demonstrated the feasibility of detecting cells with known structural abnormalities based on hybridization of breakpoint spanning YAC DNA probes in interphase cells and outlined schemes of their application in preconception/implantation genetic diagnosis. At this time,

PGD of translocations requires a major effort in developing translocation specific probes, but further progress in the Human Genome Project is expected greatly to simplify and expedite the process. This should lead to significantly lower costs, thus making interphase PGD affordable for most infertile couples considering IVF.

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