Application of Micro-FISH for Characterization of Structural Human Chromosome Abnormalities

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ABSTRACT Structural chromosome aberrations, especially *de novo* translocations and marker chromosomes, often remain uncharacterized in clinical cytogenetic analysis. Due to the limitations of conventional banding analysis in precisely identifying these rearrangements and marker chromosomes, genetic counselling is exceedingly difficult. Microdissection combined with fluorescence *in situ* hybridization (micro-FISH) has become a powerful tool in clinical genetics for the characterization of cytogenetically unclassifiable aberrations. Micro-FISH was used to elucidate the chromosomal origin of two different *de novo* structural chromosome abnormalities. Ten copies of aberrant chromosomes were collected with microneedles from patient's metaphases, transferred to a collecting drop and amplified by means of degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR). The PCR products were labeled by nick-translation with digoxigenin-11-dUTP and used as FISH probes. They were hybridized to metaphase spreads from patients to confirm the specificity of the probe and normal metaphases to determine the origin of the aberrant chromosomes. With this strategy, a *de novo* marker chromosome and a *de novo* isodicentric chromosome in peripheral blood samples were successfully identified in two cases. One marker of a small ring chromosome appeared to be derived from the pericentromeric region on the short arm and long arm (Xp11.1-q12) of the X chromosome and the second aberrant was identified as an isodicentric X chromosome (idic(X)(q28)). Based on the analysis of both G-banding and micro-FISH, the karyotypes for the patients were defined as mos 46,X,r(X)(p11.1q12)[64]/45,X[26]/46,XX[10] for the first patient and mos 46,X,idic(X)(q28)[86]/45,X[12]/46,XX[2] for the second case, respectively.

KEYWORDS: chromosome microdissection , fluorescence *in situ* hybridization, marker chromosome.

INTRODUCTION

In the past decade, fluorescence *in situ* hybridization techniques utilizing chromosome specific, centromere- specific and numerous chromosome band- specific probes have become a part of routine cytogenetic analysis for studying chromosomal rearrangements.[1] However, these techniques have a disadvantage in that probes and chromosome paints used must be tested empirically when cytogenetically unidentifiable chromosome abnormalities, such as marker chromosomes and unbalanced *de novo* translocations are involved. Meltzer et al[2] and Deng et al[3] introduced a technique based on the physical dissection of GTG - banded chromosomes followed by a degenerate oligonucleotide primed – polymerase chain reaction (DOP-PCR) and subsequent FISH using the total PCR product as a probe (micro-FISH), which overcome this limitation. Application of micro-FISH for successfully synthesizing region - and band - specific DOP - PCR products can be used in clinical cytogenetics as FISH probes for reverse painting to analyze marker chromosomes, deletions, unbalanced translocations, complex chromosome rearrangements, and other structural chromosomal abnormalities involving the dissected region.[4-8] In this report, the experiments was designed to describe the characterization of two *de novo* rearrangements in peripheral blood samples using this micro-FISH technique. Application of micro-FISH made it possible to demonstrate the chromosomal origin of a small ring chromosome, and an isodicentric chromosome. The results highlight the usefulness of the micro-FISH technique in cytogenetic diagnosis.

Materials and Methods

Clinical investigation

Patient A was an 19-year-old female referred for chromosome investigation because of growth retardation. Clinical features suggestive for Turner syndrome (TS) were shield chest, widely spaced nipples, low set ears, slanted eyes, short stature and primary amenorrhea. The patient was mentally retarded and epileptically displayed. Analysis of GTG-banded chromosomes of the patient revealed that a normal karyotype was present in 10%, only one X-chromosome was present in 26% of the cells while 64% had beside a normal X-chromosome a marker chromosome (Fig 1A). The karyotype formula was mos 46,X,+mar *de novo*/45,X/46,XX. The marker appears to be about one-third the size of a chromosome 21, lacking an identifiable G-banding pattern. The karyotypes of the parents were normal.

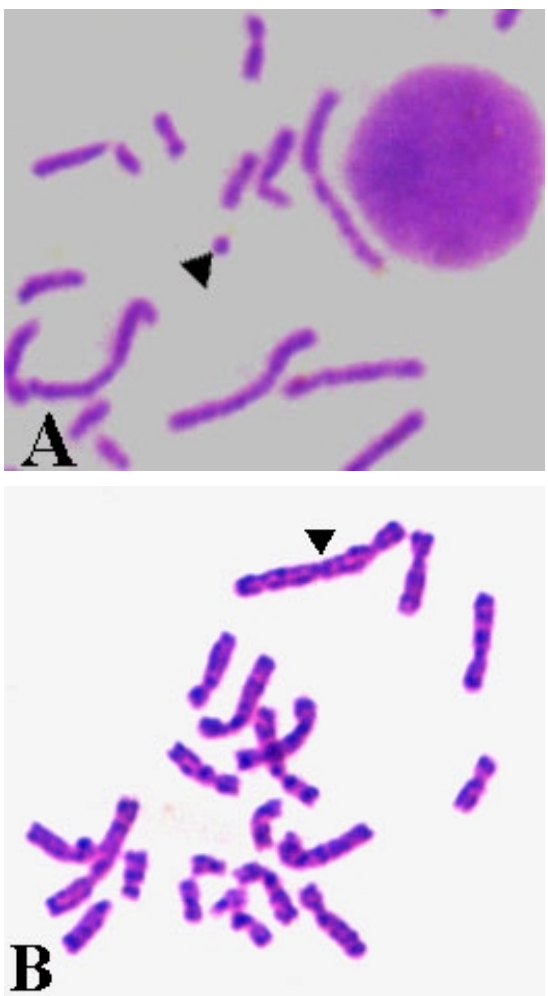


Fig 1. A) GTG-banded marker chromosome of patient A.
B) GTG-banded unbalanced translocation chromosome of patient B.

Patient B was an 17-year-old female, she was evaluated for short stature, primary amenorrhea, and lack of development of secondary sex characteristics. She did not have the classical phenotypic signs of TS. Physical examination confirmed the absence of mammary development and of pubic and axillary hair. Pelvic examination revealed well developed labial folds, a normal clitoris, and normal urethral and vaginal openings, but no cervix and uterus was palpated. Chromosome analysis showed a normal 46,XX karyotype in 2% of the cells, a 45, X karyotype in 12% and 86% of cells were 46,X,idic(X)(q28) karyotype. The size of the translocation was bigger than the chromosome 1 (Fig 1B). By GTG-banded pattern, it was identified as an isodicentric X chromosome and their breakpoints and reunion were at band q28. With C-banding technique the abnormal chromosome revealed two centromeres. Both parents' karyotypes were normal.

Preparation of metaphase chromosomes

Chromosomes were prepared from peripheral blood lymphocytes using the synchronization method of Dutrillaux and Viegas-Pequignot[9] with only minor modifications. After three days of peripheral blood lymphocyte cultivation in RPMI 1640 medium containing fetal calf serum, phytohemagglutinin (PHA) and antibiotics, and treated for overnight with 5- BrdU at a final concentration of 200 μg/ml. Then the cells were washed twice with RPMI 1640, and reincubated with complete medium (RPMI 1640 + fetal calf serum + 0.3 μg/ml thymidine) for 6 h. Cultures were exposed to colcemid (0.05 μg/ml) for 30 min prior to harvest. Chromosome spreads for microdissection were made with air dried technique according to routine procedures on 24 x 50 mm coverslips[10]. The coverslips were rinsed in distilled water and stored in 98% ethanol at –20 °C. High resolution banding was performed by digesting the chromosomes with 2.5% trypsin and followed by staining with 10% Giemsa in Sorensen's buffer (pH 7.0) for 5 min to obtain a GTG banded pattern prior to microdissection.[10]

Microdissection of Chromosomal DNA

Microdissection was performed on an inverted microscope (OLYMPUS, Olympus Optical Co.,Ltd, Shinjuku-ku, Tokyo, Japan) with the aid of a Narishige micromanipulator (MO-202, Nikon Co., Chiyoda-ku, Tokyo, Japan) and siliconized glass

needles, which had been extended with a pipette puller (Narishige Model PC-10). The minimum size of a dissected chromosome region was limited by the diameter of the needle tip which should have an diameter of about 0.3-0.5 µm. To avoid contamination, the needles and microcentrifuge tubes including the collection drop and pipettips were treated with UV light for 30 min. A prometaphase is located with well-isolated chromosome and position the target region relative to the tip of the dissecting needle before scraping the needle across the prometaphase that was covered with 1-2 µl mili-Q water (Fig 2).[11] In the experiments, 5-10 copies of the marker chromosomes from patient A and the isodicentric chromosomes from patient B were collected in 0.5 ml microcentrifuge tube containing a 20 µl collection buffer (2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1 mg/ml gelatine). A fresh microneedle was used for each dissection.

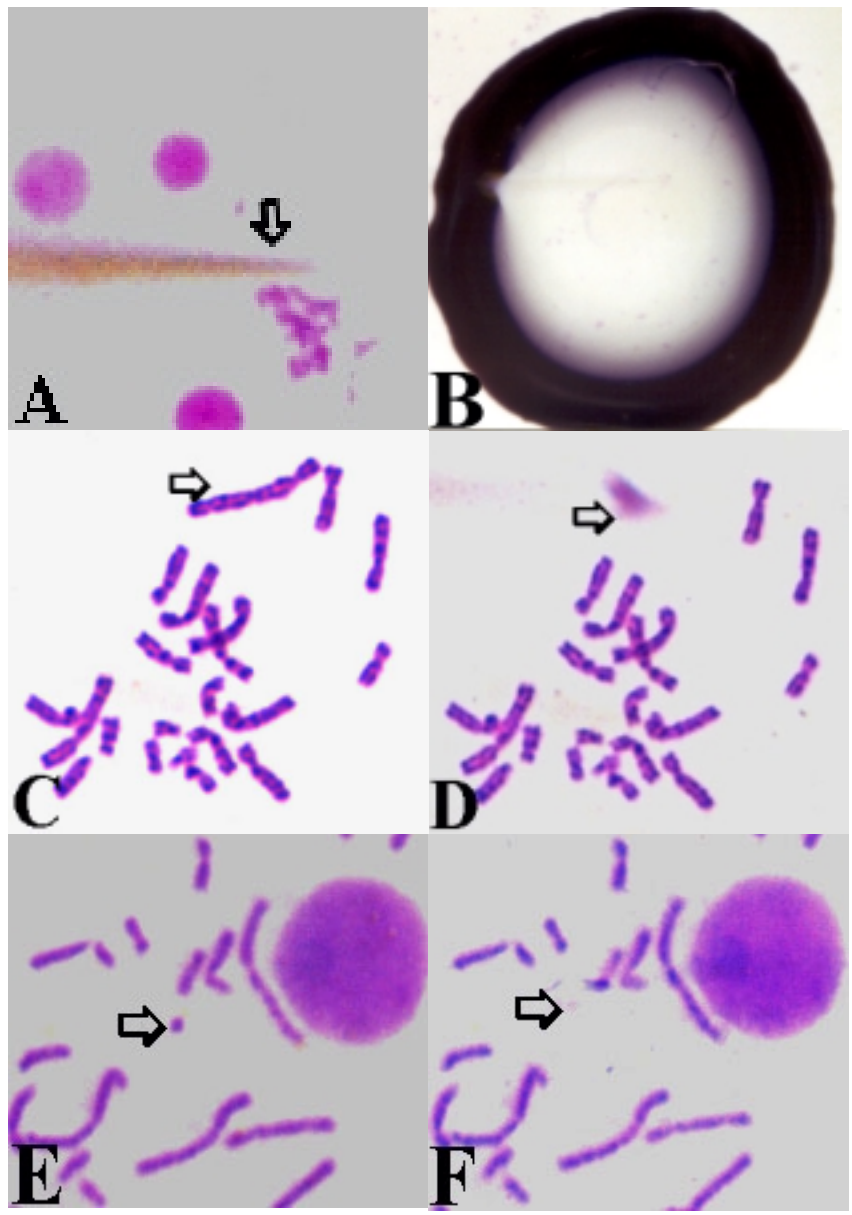


Fig 2. Procedure followed during microdissection.
A) Location of a well spread metaphase and installation of the microneedle.
B) Covering of the metaphase with Milli-Q water.
C,D) Dissection of unbalanced translocation chromosome of patient B.
E,F) Dissection of marker chromosome of patient A.

Amplification of Chromosomal DNA

After sufficient chromosomal materials were collected, the samples were cycled by DOP-PCR following the slightly modified protocol of Engelen et al.[11] The samples were cycled for 20 cycles of 30 °C for 1 min and 50 °C for 1 min in Touch Down thermal cycler (HYBAID, Thermo Hybald Co., Ashford, Middlesex, U.K.) with a heated lid. Subsequently, 30 µl PCR buffer containing 100 µM of each dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1 mg/ml gelatine, 4 µM universal primer and 1.5 U AmpliTaq Gold (Perkin Elmer, Cetus Co., Norwalk, Connecticut, U.S.A.) were added to each sample. The universal primer was first published by Telenius et al[12] with the sequence of 5′ CCGACTCGAGNNNNNNATGTGG 3′. For initial denaturation, the mixture was heated to 94 °C for 10 min and 93 °C for 3 min followed by 8 cycles of 1 min at 94 °C, 1 min at 30 °C, 1 min at 45 °C and 3 min at 72 °C and by 28 cycles of 1 min 94 °C, 1 min at 56 °C and 3 min at 72 °C with a final extension step at 72 °C for 10 min. The DOP-PCR experiments always contained a negative control consisting of all PCR components except micro-dissected DNA and a positive control with 25 pg total human DNA. The experiment was continued if DNA synthesis was not apparent in the blank reaction as determined by ethidium bromide staining of an agarose gel of the PCR products (Fig 3). The PCR products were precipitated and purified by adding 1/10 volume of 3M sodium acetate, washed with 70% ethanol and dissolved in TE buffer (10 mM Tris- HCl, 1 mM Na2EDTA, pH 8.0).

FISH

PCR products were labeled by nick- translation with digoxigenin (DIG)-11-d UTP (Boehringer, Boehringer Ingelheim GmbH, Germany). Metaphase spreads for FISH were prepared from PHA-stimulated lymphocytes using standard methanol : acetic acid (3:1) fixation.[10] FISH protocol were performed following the synchronization method of Engelen et al[11] with only minor modifications. Briefly, for hybridization, 200 ng probe was used in 10 µl hybridization mixture containing 50% formamide, 2XSSC, 10% dextran-sulphate, and 10 µg human Cot-1 DNA (BRL). Probe and Cot-1 DNA were denaturated at 80 °C for 7-8 min and reannealed at 37 °C for 90-150 min. Slides were denaturated in 70% formamide, 2XSSC (pH 7.0) at 74 °C for 4-5 min, dehydrated and hybridized with probe at 37 °C for overnight in a moist chamber. To remove excess probe, the slides were then washed three times in 2XSSC (pH 7.0) at 42 °C for 5 min, three times in 0.1XSSC (pH 7.0) at 60 °C for 5 min and three times in 0.1% tween 20 at 37 °C for 5 min. Probe detection and signal amplification were performed by applying alternating layers of anti-digoxigenin antibody conjugated with rhodamine (Boehringer) followed by three washes with 0.1% tween 20 and one wash with PBS. Slides were dehydrated and counter-stained with a 0.5 µg/ml DAPI (Sigma), followed by mounting with anti-fading reagent (Vysis, Amoco Technology Co., Illinois, U.S.A.). After FISH, cells were viewed under a Nikon EFD-3 fluorescence microscope (Nikon Co., Chiyoda-ku, Tokyo, Japan) and Olympus BX60 fluorescence microscope with image analyser (Applied Imaging, Olympus Optical Co., Ltd, Shinjuku-ku, Tokyo, Japan). Photographs were taken on Kodak color slide film (ASA 100).

RESULTS

Case 1

Reverse chromosome painting to normal metaphases with a marker-derived from small ring probe pool resulted in strong signals in the pericentromeric region of two chromosomes of the

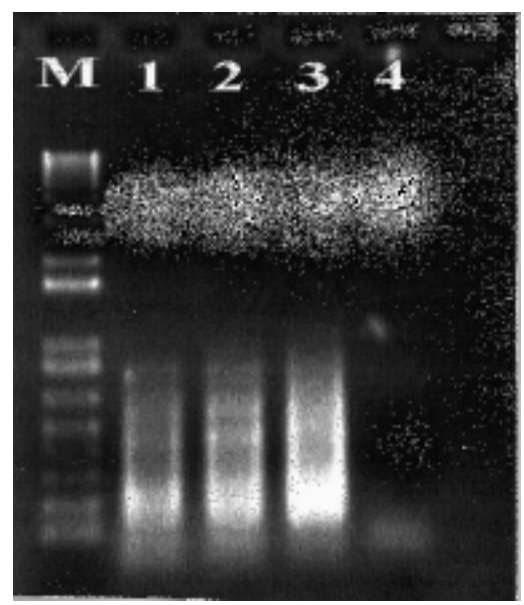


Fig 3. Ethidium-bromide-stained agarose gel demonstrating the products of DOP- PCR of five microdissected chromosome fragments. Lane M = 1 kb plus DNA marker. Lane 1 = DOP-PCR products of five microdissected chromosomal DNAs from the marker chromosome of patient A. Lane 2 = DOP-PCR products of five microdissected chromosomal DNAs from the isodicentric X chromosome of patient B. Lane 3 = positive control (25 pg of DNA). Lane 4 = negative control amplified with no template DNA.

C-group. After analysis of the DAPI counterstained chromosomes it became clear that the chromosomes involved were the X chromosomes (Fig 4 A-B). FISH with the same probe to the patient's metaphase chromosomes confirmed this results showed a strong signal in the pericentromeric region on the short arm and long arm (Xp11.1-q12) of the normal X chromosomes, as well as the entire ring chromosome (Fig 4 C-D). Based on the analysis of both G-banding and micro-FISH, the karyotype for the patient A is therefore defined as mos 46,X,r(X)(p11.1q12)[64]/45,X[26]/46,XX[10] (Fig 5 A).

Case 2

A probe pool made from the entire aberrant isodicentric X chromosome in patient B was hybridized to metaphase chromosomes of a karyotypically normal person. FISH-positive sites were assigned to the distal regions of the short arm of chromosome X up to the band q28 (Fig 6 A-B). This suggested that the origin of this aberrant was chromosome X. Consequently the resulting karyotype, mos 46,X,idic(X)(q28)[86]/45,X[12]/46,XX[2] (Fig 5 B). Furthermore, FISH with the same probe to the patient metaphase chromosomes confirmed this result and revealed a paint of the whole isodicentric X chromosome. In conclusion, the results of micro-FISH confirmed the diagnosis by conventional GTG-banding technique that the chromosome was defined as isodicentric X chromosome with no other chromosome material (Fig 6 C-D).

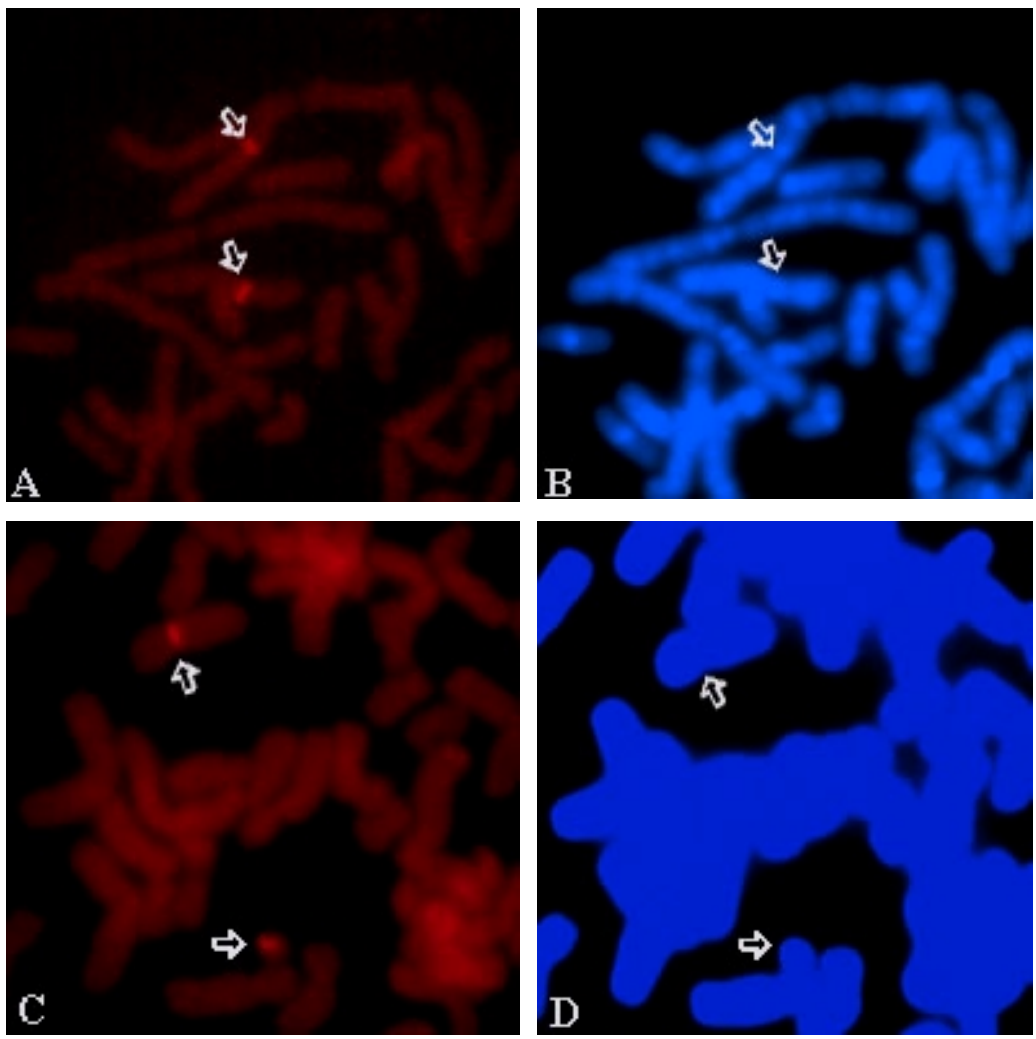
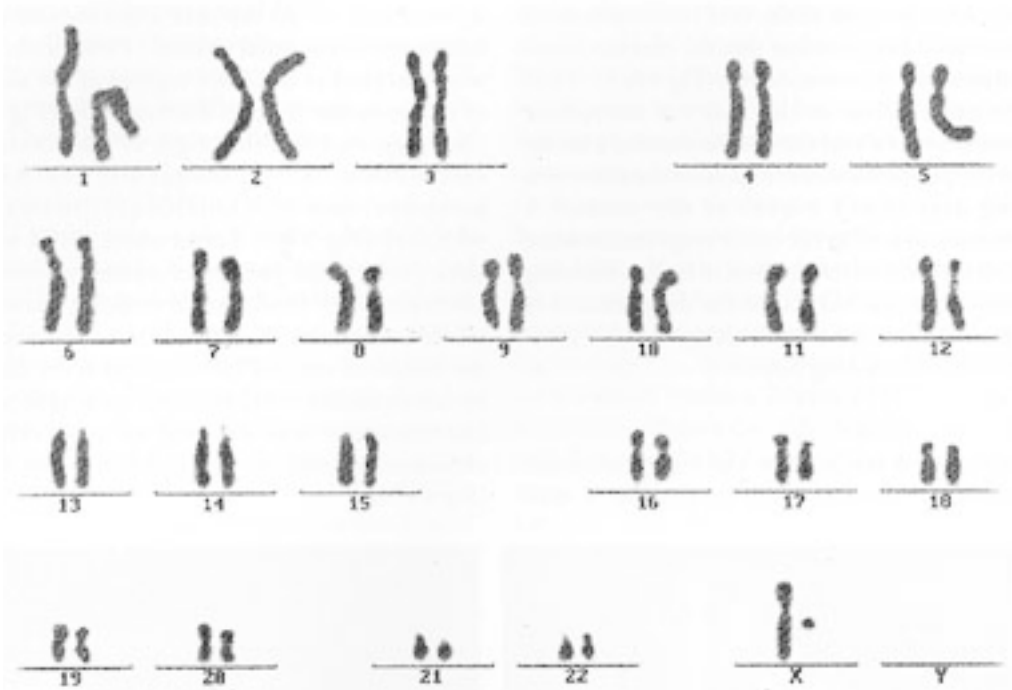


Fig 4. A) FISH with the DOP-PCR probe from patient A on a metaphase of a normal woman. Probe hybridized on the Xq11-12.
B) DAPI counterstaining of A.
C) DOP-PCR probe from patient A hybridized to a metaphase of patient A.
D) DAPI counterstaining of C.



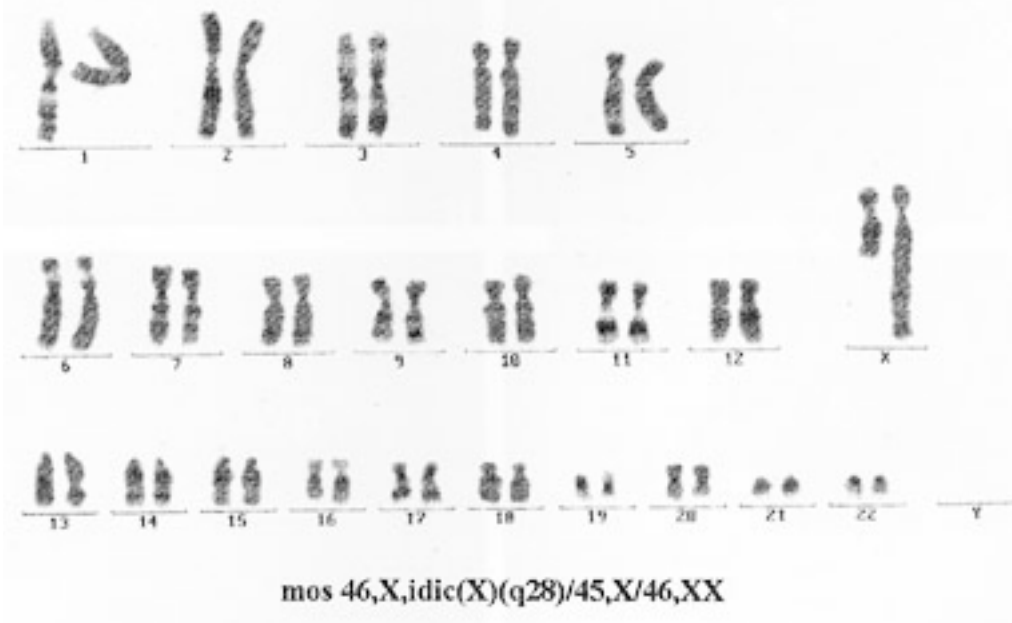


Fig 5. GTG banded karyotype from the patients.
A. A karyotype of patient A.
B. A karyotype of patient B.

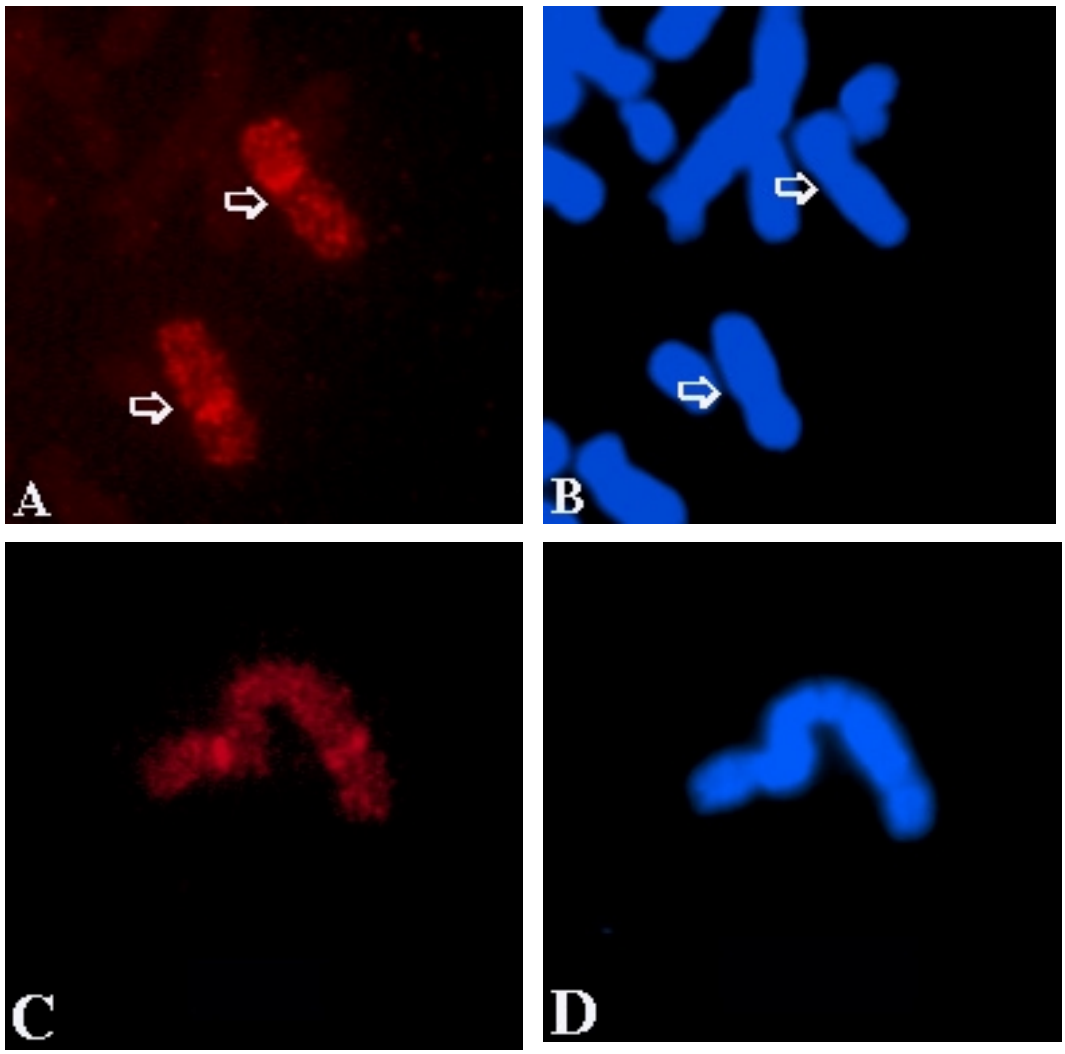


Fig 6. A) FISH with the DOP-PCR probe from patient B on a metaphase of a normal woman. Probe hybridized on the whole length of the X chromosomes.
B) DAPI counterstaining of A.
C) DOP-PCR probe from patient B hybridized to a metaphase of patient B.
D) DAPI counterstaining of C.

Discussion

The present study which has demonstrated the combined strategy of chromosome microdissection, DOP-PCR and reverse chromosome painting (micro-FISH) is a powerful method in clinical cytogenetics. When a marker-specific micro-FISH probe is generated and used for reverse painting, one single step of hybridization gives detailed information of the chromosomal composition of the marker. In addition to revealing the chromosome(s) involved in the rearrangement, micro-FISH can also pinpoint the precise chromosomal region(s) contained within the marker. For our patients, the most direct way to identify the small ring marker chromosome in patient A is the micro-FISH approach as no clue is available from G-banding analysis regarding the origin of the ring chromosome. In patient B, although the idic(X) was strongly suggested by the G-banding pattern, unequivocal identification of this structural rearrangement and exclusion of the involvement of X were only achieved by reverse painting with the micro-FISH probe generated from the marker chromosome itself. Patient A has a small ring marker that is derived from the X chromosome containing the region Xp11.1 to Xq12. The karyotype for patient A is therefore defined as mos 46,X,r(X)(p11.1q12)[64]/45,X[26]/46,XX[10].

Since there is quantitative disparity between the sex with respect to the gene loci on human X and Y chromosomes, a mechanism for dosage compensation between females and males has evolved in order to achieve genetic equivalency between the two. The X chromosome inactivation is a developmentally regulated process by which dosage compensation occurs and is governed by the X inactivation center which contains the XIST gene (X - inactive – specific transcript).[13] Mental retardation, severe short stature, and other malformations are not normally associated with TS patients having a 45, X karyotype. These atypical features in TS correlate with the presence of small r(X) chromosomes and may be due to the absence or malfunction of the XIST gene.[14-16] The XIST locus, residing in the region of the putative X inactivation center (XIC) in Xq13.2 is expressed only from the inactive X chromosome and is considered a prime player in the initiation of mammalian X inactivation.[17,18] The abnormal phenotype and/or mental retardation seen in patients with a small marker X chromosome results from a deletion of the XIC and from the resultant functional disomy of pericentromeric sequences.[17] The phenotypic picture of our patients with a small r(X) included mental retardation and growth retardation more severe than expected in TS and it occasionally included seizures, probably due to the absence of the X-inactivation center in the marker. Therefore, our data support the hypothesis that the X-inactivation center was lost, then inactivation probably never occurred and cells bearing the ring would have two active copies of each gene on the ring. As with autosomal duplication, this chromosomal imbalance could result in mental retardation. However, one previous report provides considerable evidence against it. The results suggested that mental status in females with r(X) chromosomes was determined by multiple factors including the presence or absence of XIST on the r(X) chromosomes, the size and frequency of active r(X) chromosomes, in addition to co-incidental genetic and environmental factors.[19] In patient B, a mos 46,X,idic(X)(q28)[86]/45,X[12]/46,XX[2] karyotype was found by cytogenetic analysis and confirmed with micro-FISH. Her clinical features correspond well with the previous studies. In 1982, Robertson et al reported an isodicentric X chromosome (46,X,idic(X)(pter → qter::qter → pter)) in a moderately tall patient with gonadal dysgenesis.[20] In 1987, a 17-year-old female patient with gonadal dysgenesis but no other turnerian features was found to have a 46, X, idic(X)(q2803) karyotype in her lymphocytes.[21] An isodicentric X-chromosome, idic(X)(pter → q26.1:: q26.1 → pter), was found in lymphocytes and ovarian tissue of a 40-year-old female patient with secondary amenorrhea.[22] In 1993, there were two cases of dicentric isochromosome X with premature ovarian failure and gross appearance did not present an obvious picture of TS, except for short stature, poor development of secondary sexual charactersitic and infertility.[23] Isodicentric X chromosomes are formed by the fusion of two X chromosomes. The phenotypic effects are variable and depend on whether the chromosomes are fused at long or short arms, and whether or not there is a deletion. Patients with isodicentric X chromosomes joined at their short arms exhibit short or normal stature, gonadal dysgenesis, and occasionally TS features, whereas those with long arms joined are normal or above average in stature and have gonadal dysgenesis.[24] The cause of the breakpoints and the parental origin of these dicentric chromosomes is uncertain. Several mechanisms have been proposed to explain the formation of dicentric X chromosomes. In our patient, the origin of the dicentric X chromosome probably was the chromatid breakage, with subsequent reunion of the sister chromatids and splitting of the centromere; the resulting chromosome may be referred to as isodicentric and consists of duplicate portions of a single X chromosome with symmetric banding patterns at opposite sides of the breakpoint.[25] The stability of dicentric chromosomes is presumably a result of centromere suppression and associated premature centromere division of the suppressed centromere.[26] The isodicentrix X is almost always late replicating. The second centromere is nonfunctional, making it a pseudodicentric chromosome.[24]

Reverse chromosome painting not only works efficiently for the confirmation and refinement of abnormal karyotypes, but, more importantly, also works for the elucidation of small and complex chromosome rearrangements and marker chromosomes of unknown origin. This strategy offers a very reliable approach to rapidly establishing more accurate genotype-phenotype correlations as a prerequisite for comprehansive genetic counselling.

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