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Short Communication

CHARACTERIZATION OF MARKER CHROMOSOMES BY FISH USING MICRODISSECTED PROBES FROM OLD CARNOY-FIXED CELLS: REPORT OF TWO CASES

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Summary We reported on two patients with a *de novo* marker chromosome of which the origins were successfully identified by FISH using microdissected probes. These probes were established by microdissections of extra chromosomal segments from Carnoy-fixed cells stored at −20°C for several years. Using these probes, we could verify partial 1q32 trisomy in a patient with 17p+ as well as partial 16q2 trisomy in another patient with 4p+.

Key Words microdissection, fluorescent *in situ* hybridization (FISH), marker chromosome, *de novo* chromosome rearrangement

Introduction

It is difficult to know the origin of a *de novo* marker chromosome by conventional G- or R-banding techniques, especially when its extra segment is too short to compare the banding pattern or banding pattern is nonspecific. This is a reason why most patients with these chromosomal abnormalities are provided an inadequate genetic counseling, even though they have multiple congenital anomalies and mental retardation.

Recently, a microdissection technique of chromosome and subsequent amplification of the dissected chromosomal segments by polymerase chain reaction (PCR) have been developed. Three different techniques have been designed to amplify DNA templates from the dissected chromosome; (1) linker adapter-PCR (LA-PCR) (Lüdecke *et al.*, 1989; Deng *et al.*, 1992; Hirota *et al.*, 1992), (2) degenerate oligonucleotide-primed PCR (DOP-PCR) (Guan *et al.*, 1992; Meltzer *et al.*, 1992; Telenius *et al.*, 1992), and (3) DOP-Shuttle-PCR (Yokoyama and

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Sakuragawa, 1995). By using the PCR products as a probe pool, fluorescence *in situ* hybridization (FISH) combined with chromosome *in situ* suppression (CISS) hybridization may characterize the nature of chromosomal rearrangements. These methods have been applied for precise definition of an interstitial deletion (Rubtsov *et al.*, 1996), a breakpoint region in human B-cell lymphoma (Guan *et al.*, 1996), prenatal diagnosis of a marker chromosome (Müller-Navia *et al.*, 1995), and characterization of a marker chromosome or translocations (Deng *et al.*, 1992; Ohta *et al.*, 1993; Ohashi *et al.*, 1994; Müller-Navia *et al.*, 1995; Eggermann *et al.*, 1996; Engelen *et al.*, 1996). Here we reported on two patients with a marker chromosome identified its origins by FISH using a microdissected probe pool established from Carnoy-fixed cells stored at −20°C for several years.

Materials and Methods

Clinical reports and cytogenetics. Patient A was 2-year-old girl with severe psychomotor retardation, failure to thrive, and multiple congenital defects. She had dysmorphic facies with large fontanelle, frontal bossing, deep-set eyes, posteriorly rotated and low-set ears, bilateral cleft lips, cleft palate and micrognathia. Other findings included limited extension at the elbows, delayed bone age, Dandy-Walker malformation, and left hydronephrosis.

Patient B was referred to us at 3 years of age for psychomotor and growth retardation and a marker chromosome 4p+. She had mildly triangular facies with frontal bossing, telecanthus, downslanting palpebral fissures, iris coloboma, high and wide nasal bridge, wide philtrum, and short neck. Her extremities showed limited pronation and spination at the wrists, limited extension at the elbows, brachyclinodactyly of fifth fingers, and large halluces.

Chromosomal analyses of the patients and their parents were performed on phytohemagglutinin-stimulated lymphocytes using standard G-banding techniques.

Microdissection and amplification of dissected DNA. The procedure for chromosome microdissection was performed essentially as described previously (Deng *et al.*, 1992; Hirota *et al.*, 1992; Ohta *et al.*, 1993). Briefly, for preparation of metaphase chromosomes, Carnoy-fixed (methanol : acetic acid = 3 : 1) cell suspensions stored at −20°C for 1 1/2 years (patient A) and 4 1/2 years (patient B) were spread onto each clean glass-slide. Immediately the slides were washed in phosphate buffer (pH 6.8) for 10 min, and were rinsed in two changes of 70% ethanol. GTG-banding was performed with 0.0005% trypsin and 3% Giemsa at room temperature. Thirty pieces of the target chromosome were microdissected with a fine glass needle under an inverted microscope. They were transferred into a collection chamber and were covered with paraffin oil. Further steps, including proteinase K digestion, DNA extraction, Sau3A1 digestion, DNA ligation to a linker/primer set, and PCR, were carried out as described previously (Hirota *et al.*, 1992). Takara Ex *Taq* (Code No. RR001A) was used as a DNA polymerase.

The size of the PCR products ranged from 200 to 700 bp.

Fluorescence in situ hybridization (FISH). The PCR products from the dissected target chromosome were labeled with biotin-11-dUTP by a secondary PCR as described previously (Ohta *et al.*, 1993). After an addition of 10 μg Salmon sperm DNA (D-1626, Sigma), and 5 μg human Cot-1 DNA (5279SA, Gibco BRL) as a competitor into a 1/20 volume of the second PCR product, the DNA mixture was ethanol-precipitated, resuspended in 10 μl formamide, denatured at 75°C for 10 min, and mixed with a hybridization solution consisting of 20 mg/ml bovine serum albumin, 10×SSC and 50% dextran sulfate in a volume ratio of 1 : 2 : 2. The hybridization mixture was incubated at 37°C for 30 min, and was put onto denatured chromosomes. Overnight hybridization was performed at 37°C. Further steps, including washing, incubation with fluorescence isothiocyanate (FITC)-conjugated avidin, counterstaining with propidium iodide (PI), were carried out as described previously (Ohta *et al.*, 1993). Photomicroscopy was performed under a fluorescence microscope equipped with a B-2A filter (Nikon).

Results

Patient A. On the analysis of GTG-banded chromosomes, one chromosome 17 had an extra segment on the short arm. Karyotypes of the parents were normal. Her karyotype was designated as 46,XX,17p+ (Fig. 1a). With a probe pool established from 30 dissected fragments of 17p+, FISH on control metaphases painted distal fourth regions of chromosomes 1q as well as terminal segments of normal 17p (Fig. 2a). To ensure these results, with a probe pool established from microdissected fragments of the distal half region of normal chromosome 1q, FISH on the patient metaphases painted distal half regions of chromosomes 1q and 17p+, but not normal 17p (Fig. 2b). As a result of G-banding pattern and FISH analyses, we concluded that she has partial trisomy for 1q32-qter.

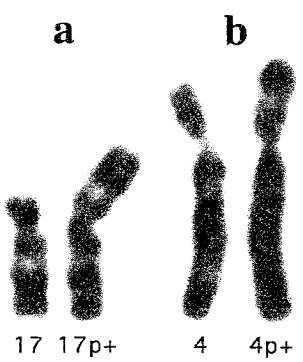

Fig. 1. a: A GTG-banded marker chromosome of patient A. b: A GTG-banded marker chromosome of patient B.

Patient B. Cytogenetic analysis of GTG-banded chromosomes confirmed an extra band on 4p (4p+) (Fig. 1b). With a probe pool from 25 dissected fragments of chromosome 4p+, FISH on control metaphases showed signals in distal regions of chromosomes 16q and almost all segments of chromosomes 4p (Fig. 2c). With a reverse probe pool made from microdissected fragments of normal 16q including a candidate region, FISH on the patient metaphases showed painting of normal 16q and an interstitial region of 4p+ (Fig. 2d). As a result of G-banding pattern and FISH analyses, we concluded that she might have partial trisomy for a part of 16q22-qter, which probably is inserted into the short arm of chromosome 4.

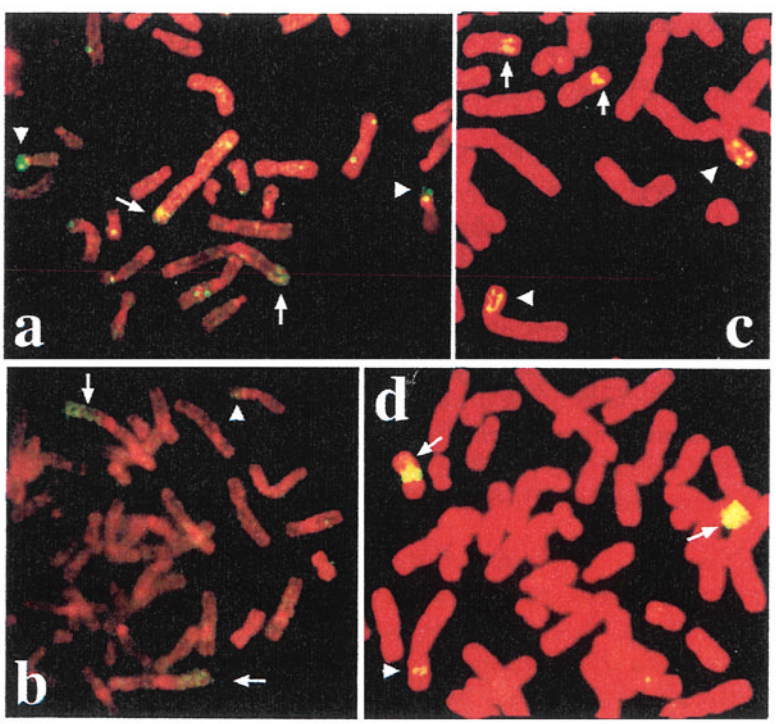

Fig. 2. a: FISH with microdissected probes from 17p+ on normal metaphases. FITC signals painted distal fourth regions of chromosomes 1q (arrows) as well as normal 17p (arrowheads). b: FISH with microdissected probes from the distal half region of normal chromosome 1q on patient A metaphases. FITC signals painted distal half regions of chromosomes 1q (arrows) and a distal region of 17p+ (arrowhead). c: FISH with microdissected probes from 4p+ on control metaphases. FITC signals painted both distal regions of chromosomes 16 (arrows) and almost all segments of chromosome 4p (arrowheads). d: FISH with microdissected probes from the long arm of normal chromosome 16 on patient B metaphases. FITC signals painted both normal 16q (arrows) and an interstitial region of 4p+ (arrowhead).

Discussion

It has been shown that microdissected probes from Carnoy-fixed cells did not hybridize well in FISH analyses (Viersbach *et al.*, 1994). On this poor hybridization it was suggested that depurination of DNA could occur during methanol-acetic acid fixation (Holmquist, 1979). The depurinated sites are labile and may form nicks. And the size of DNA isolated from fixed preparations after aging for 24 hr or more was diminished (Mezzanotte *et al.*, 1988). Thus, to avoid depurination by acetic acid, Epstein-Barr virus-transformed lymphoblastoid cells or peripheral blood lymphocytes harvested in 100% methanol fixation were preferred to store at −20°C until use, and a fixed cell suspension was mixed with acetic acid immediately prior to spread onto a clean glass-slide (Deng *et al.*, 1992; Ohta *et al.*, 1993; Ohashi *et al.*, 1994).

However, recently, several reports using fixed cell suspensions by Carnoy's solution (methanol : acetic acid 3 : 1) have been published with minor differences. Carnoy's fixation was performed within 2 hr before making slides and metaphase spreads were stored at 37°C for 2–3 days (Guan *et al.*, 1992). Recently, more impressive result was reported by Engelen *et al.* (1996). They used conventionally Carnoy-fixed cell suspensions stored at −20°C for 3, 5, and 10 months. And chromosome preparations from Carnoy-fixed cells were stored in 98% ethanol at −20°C until use. Furthermore, Li *et al.* (1995) reported on quality of DNA templates from Carnoy-fixed cells. They showed that there were no differences in quality of amplification products between PCR templates obtained from fresh tissues or from Carnoy-fixed cells for varying length of time ranging from a few weeks to 6 years, and that there was no apparent degradation of the DNA from Carnoy-fixed cells stored at −20°C. The results of present study confirmed these data, because we could successfully make the probes from stored Carnoy-fixed cell suspensions at −20°C for 1 1/2 and 4 1/2 years.

Successful painting by probes made from Carnoy-fixed cells may be attributed to increased efficiency of PCR by Ex *Taq* polymerase used in this study. The Ex *Taq* polymerase formulation consists of a combination of a high level of an exonuclease-free *Taq* polymerase with a low level of a thermostable DNA polymerase exhibiting a 3′-exonuclease activity (TaKaRa Shuzo Company). Polymerase combinations of this type could amplify long targets with high fidelity (Barnes, 1994; Cheng *et al.*, 1994; Ermak *et al.*, 1996).

FISH using microdissected probes is a useful technique for identification of the origin in cases with *de novo* chromosomal rearrangement. As indicated in this report, microdissected probes from old Carnoy's fixed cells were available for FISH analyses as same as from fresh cells. Thus, an application of this technique for marker chromosomes, in which its origins were not identified in the past trials, would support further genetic information to the patients with a marker chromosome.

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