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THE APPLICATION OF MICRO-FISH IN CLINICAL CYTOGENETICS

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THE APPLICATION OF MICRO-FISH IN CLINICAL CYTOGENETICS

PROEFSCHRIFT

**ter verkrijging van de graad van doctor aan
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Chapter 1

GENERAL INTRODUCTION

Chromosome banding

Clinical cytogenetics is the field of research that studies chromosomes at the microscopic level, with the aim to detect chromosome abnormalities and to explain their significance for the phenotype. Currently, Giemsa banding is the most widely used chromosome identification technique. It involves trypsin treatment of metaphase chromosome preparations followed by staining with Giemsa. This results in a pattern of light and dark bands that is unique for each chromosome. With this technique, 350-550 bands for a haploid set of human chromosomes can be observed. This means that one band represents on average approximately $5-10 \times 10^6$ bp of DNA. Examination of chromosomes in prometaphase, at which stage the chromosomes are less condensed than in metaphase, allows even higher degrees of resolution. Not less than 850 bands or even more than 1000 bands per haploid set of human chromosomes can be obtained in prometaphase.

Chromosome abnormalities

Chromosome abnormalities may be numerical or structural. Numerical chromosome abnormalities involve the gain or loss of one or more chromosomes (aneuploidy) and sometimes the gain of a complete set of chromosomes (polyploidy). Structural chromosome abnormalities may involve one or more chromosomes. When only one chromosome is involved, a part of the chromosome may be lost (deletion) or may be present twice (duplication). Furthermore, a part of one chromosome can be inverted with the centromere involved (pericentric) or not involved (paracentric) in the inversion. Structural abnormalities involving two or more chromosomes may result in insertion(s) of material of one chromosome into another, or translocation(s) of chromosomal material between two or more chromosomes. Individuals carrying a balanced translocation (either reciprocal or Robertsonian) are genetically and clinically normal if no chromosomal material is lost and no genes are damaged by the breakage of the chromosomes. However, such individuals are at increased risk of having chromosomally unbalanced offspring.

***In situ* hybridization**

During the last fifteen years *in situ* hybridization (ISH) has been introduced as a diagnostic tool in clinical cytogenetics. This technique, first described in 1969 by Gall and Pardue¹, can give information about the presence and expression of specific nucleic acid sequences in dividing and non-dividing cells. For ISH, a DNA fragment referred to as a probe, is labeled with a reporter molecule. After hybridization with cellular DNA the probe hybridizes with homologous nucleic acid sequences and can be detected. The detection procedure depends upon the nature of the reporter molecule. In the past, radioactive labeled probes were detected by autoradiography. Nowadays the reporter molecules are immunochemically detected by an antibody coupled with a

fluorochrome or the reporter molecule is a fluorochrome itself. For the analysis of chromosome aberrations with fluorescence *in situ* hybridization (FISH) four major groups of probes are at the moment available.

- [1] **Whole chromosome painting probes** paint a chromosome more or less completely and can be used for the analysis of chromosome translocations and insertions.
- [2] **Chromosome specific repetitive sequence probes** hybridize near the centromere and can be used to determine the copy number of a chromosome in a non-dividing cell or the centromere that is present in a Robertsonian translocation chromosome.
- [3] **Unique sequence probes** are used to study specific aberrations like deletions and duplications or breakpoints in a translocation.
- [4] **Subtelomeric probes** are localized close to the telomeres and are used to detect deletions or cryptic translocations.

Despite these technical advances there still are chromosome aberrations (like marker chromosomes and *de novo* unbalanced translocations) that cannot (easily) be identified. For the characterization of these aberrations micro-FISH has been developed.

Micro-FISH

Micro-FISH comprises the physical dissection of (GTG)-banded chromosomes using a dissection needle or a laser beam. This is followed by a degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) to amplify the dissected DNA and by labeling of the PCR product obtained with a reporter molecule. Subsequently, the DOP-PCR product is annealed to metaphase spreads from normal subjects and hybridization detected using FISH. Nowadays, micro-FISH is used in clinical cytogenetics to identify marker chromosomes and unbalanced translocations and to untangle complex chromosome rearrangements. In tumor cytogenetics micro-FISH is also used to identify the content of double minutes and homogeneously staining regions. For molecular geneticists, microdissection of chromosome regions or bands and subsequent cloning is an important step towards the isolation of disease related genes.

In 1981 microdissection was first successfully used by Scalenge et al.² for cloning genomic sequences from *Drosophila* salivary gland polytene chromosome bands. A glass needle was attached to a micromanipulator to cut chromosome segments from Section 3 of the *Drosophila* X-chromosome which were then placed in a microdrop of liquid paraffin for subsequent cloning under the microscope. From the dissected DNA, 80 phage clones were obtained which hybridized *in situ* to the chromosomal region of dissection.

Microdissection and molecular genetics

Microdissection and microcloning of mammalian chromosomes was successfully applied using mouse chromosomes in 1984 by Rohme et al.³ and human chromosomes in 1986 by Bates et al.⁴. Since no PCR technology was available at that time, a large

number of chromosome fragments (100-200) had to be dissected prior to microcloning³⁻¹². The number of clones obtained in these libraries was far from being sufficient to represent the complete dissected region. However, the clones could be used as starting points for chromosome walking and jumping to the genes of interest. The first application of this approach allowed the isolation of useful clones from the proximal half of mouse chromosome 17 by Rohme et al.³. Later the same group dissected 270 mouse chromosome segments and produced 212 microclones which served as molecular probes for isolating additional DNA sequences for fine structure analysis and mapping of the mouse t-complex region¹³.

For the microdissection of mammalian chromosomes, unstained chromosome preparations were used to avoid cloning problems associated with chromosome banding and staining procedures. As identification of unbanded chromosomes is difficult, somatic cell hybrids containing a single human chromosome were used^{10,12}. The dissection of the short arm of human chromosome 2 by Bates et al.⁴ was based on the characteristic morphology of this chromosome, being the largest submetacentric chromosome. The average insert size in the microclones derived from chromosome 2 was small, viz. 380 bp., suggested to be due to the acid treatment during fixation which results in hydrolysis of the DNA¹⁴. Later, care has been taken to minimize acid damage and microclones with larger inserts (mean length 3.2 kb.) were then obtained from a chromosome 7q22-q23 library¹⁵. Although successful, microdissection followed by bacterial microcloning has a number of drawbacks: many (100-200) chromosome fragments have to be dissected, unstained chromosome preparations are to be used and the resulting libraries do not completely represent the dissected region.

PCR amplification of microdissected material

In 1989, Lüdecke et al.¹⁶ described an improved procedure for microcloning. Trypsin-banded and Giemsa stained chromosome preparations were used to collect a smaller number of chromosome fragments (20-30) which were amplified using the PCR technique. This enabled the construction of large libraries containing tens of thousands of microclones. In this procedure, the dissected fragments were collected in a microdrop containing proteinase K and the DNA was extracted from this drop with phenol. The purified DNA was cleaved with *Rsa*I, blunt-ligated to a *Sma*I-cut pUC vector and cleaved again with *Sma*I. After amplification by PCR the amplified sequences were cloned into pUC13. Using this procedure, the dissection of only 37 fragments resulted in a chromosome 8q23-q24 library which contained 20.000 microclones. The microdissection procedure was further improved by Senger et al.¹⁷. Libraries constructed this way were used to study region 15q11-q13 in Prader-Willi and Angelman syndrome¹⁸, region Xq27-q28 in fragile-X syndrome¹⁹, region 11p13 in Wilms tumor/aniridia²⁰, region 22q12-q13 in neurofibromatosis²¹, region 5q21-q22 in adenomatous polyposis coli^{22,23} and region 8q23-q24 in Langer-Giedeon syndrome²⁴. To construct these libraries, generally 20-40 fragments were dissected and the resulting libraries contained 5,000-25,000 microclones. The average insert size ranged from 150 to 220 bp and single copy microclones represented 39-80% of the library. Most of

the clones isolated from the libraries were positive after hybridization to human DNA, and nearly all human-derived clones (>90%) were shown to originate from the dissected region²⁵. In another PCR-mediated microcloning procedure, described by Kao and Yu²⁶, sticky-end ligation of the dissected sequences to a linker-adaptor is used and followed by PCR amplification using the adaptor sequence as primer. This procedure has been applied to human chromosomes and a very large library for human chromosome 21 was constructed which contained 700000 recombinant clones. The initial steps of this procedure were performed in nanoliter volumes under the microscope. First, proteins in the microdissected fragments were digested with proteinase-K, next DNA was extracted with phenol and cleaved with MboI. Subsequently, the DNA fragments were ligated to a MboI linker-adaptor and the sample was transferred to an Eppendorf tube for PCR using the 20-nt sequence of the adaptor as a primer. The PCR products were cleaved with MboI, purified to remove adaptor molecules, cloned into pUC19, and transferred into a bacterial host. Using this procedure, a whole chromosome 21 library has been constructed from 30 dissected chromosome fragments. Thus isolation of genes was facilitated not only for Down syndrome, but also for other disorders linked to various regions of this chromosome, like Alzheimer disease, one form of epilepsy, and mental retardation²⁷.

Microdissection and clinical cytogenetics

In 1992, Meltzer et al.²⁹ introduced a novel procedure, termed micro-FISH, for the rapid generation of region-specific genomic clones from dissected chromosomal material using degenerate oligonucleotide primed PCR (DOP-PCR). DOP-PCR was developed by Telenius et al.²⁸; it consists of a PCR using a 22 bp oligonucleotide with six degenerate bases close to the 3' end (5'-CCGACTCGAGNNNNNNATGTGG-3') to generate whole chromosome paints from flow sorted chromosomes. Degenerate oligonucleotide primers allow the direct amplification of dissected DNA by priming at sites present in the genomic sequence. DOP-PCR eliminated the need for microchemical manipulations prior to PCR, because known sequences no longer had to be ligated to the microdissected material. The amplification procedure for the dissected sequences is simple in operation. The dissected fragments are transferred directly into a tube and treated with proteinase-K. Then the DNA is amplified in a first DOP-PCR reaction and biotin labeled in a second PCR reaction. The PCR products thus generated appear to be useful for chromosome painting to aid in cytogenetic analysis. Guan et al.³⁰ further improved the micro-FISH technique and introduced the use of topoisomerase instead of proteinase-K to unwind the supercoiled DNA of the dissected fragments. Furthermore, T7 DNA polymerase (Sequenase) was used in the first 8 cycles of the DOP-PCR amplification, when the annealing temperature was low. T7 DNA polymerase was chosen for this step because it functions well at the low temperatures at which the random priming complexes are stable and because it possesses strand displacement synthesis capabilities. Strand displacement synthesis enables the enzyme to synthesize long stretches of DNA by displacing other primers that have already annealed to the DNA (Bohlander et al.³¹). These methodological

improvements enabled synthesis of sufficient PCR product for chromosome painting from only a single dissected chromosome³⁰. Sequenase however, is heat inactivated at the denaturation temperature of 94 °C and subsequently active Sequenase has to be added 8 times. Consequently, these steps are a prime target for contamination.

Fig. 1: Schematic representation of the micro-FISH procedure

3. Labeling of the generated DNA with a reporter molecule.

4. Hybridization of the probe on normal metaphase chromosomes or chromosomes of the patient.

5. Immunochemical detection of the reporter molecule followed by microscopic and photographic analysis.

Clinical applications

In clinical cytogenetics micro-FISH has been successfully used to identify unbalanced chromosome translocations, marker chromosomes and complex chromosome rearrangements. Viersbach et al.³² used micro-FISH to characterize a marker in a boy with a 46,X,+mar karyotype and demonstrated that the marker was a X/Y translocation chromosome. Furthermore, they dissected two marker chromosomes in a child with a 48,XY,+mar1,+mar2 karyotype. Reverse chromosome painting revealed that the centromeres of the chromosomes 13 and 21 displayed fluorescent signals; that these chromosomes were represented in the markers was confirmed by FISH using a repetitive DNA probe specific for the centromeres of chromosomes 13 and 21, which gave positive signals on both marker chromosomes. A phenomenon always seen when micro-FISH includes the short arm of an acrocentric chromosome is that the short arms of (most of) the acrocentric chromosomes display fluorescent signals after reverse painting with the DOP-PCR product. This cross-hybridization was first reported by Viersbach et al.³² and later confirmed by Sun et al.³³ who characterized a marker derived from chromosome 21. We had the same experience in our own experiments as illustrated in fig. 2. Highly repetitive β satellite DNA present in the short arm of the acrocentric chromosomes might be responsible for this cross-hybridization (Waye and Willard³⁴).

Fig. 2: Reverse painting with DOP-PCR material derived from an isochromosome 22p showing cross-hybridization with most of the acrocentric chromosomes. D: chromosomes 13-15. G: chromosomes 21 and 22.

Besides for the identification of marker chromosomes, micro-FISH is often used for the disclosure of unbalanced chromosome translocations. In the article where Meltzer et al.²⁹ first described micro-FISH, the technique was applied on chromosomes of a patient with an unbalanced karyotype who appeared to have a partial trisomy of chromosome 21. Guan et al.³⁵ generated whole chromosome painting probes with a slightly modified method which they subsequently used to identify two complex chromosome rearrangements. Rubtsov et al.³⁶ used micro-FISH to study a terminal deletion of chromosome 6q and, unexpectedly, the hybridization pattern on normal metaphase chromosomes revealed an interstitial deletion with breakpoints in 6q25.1 and 6q27 instead of a terminal deletion.

In prenatal diagnosis micro-FISH was successfully used by Thangavelu et al.³⁷ to characterize a marker chromosome derived from chromosome 14 and by Muller-Navia et al.³⁸ who disclosed that three prenatally detected markers were derived from chromosome 3 (p12-cen), chromosome 9 (p12-cen) and chromosome 18 (pter-cen) respectively. Muller-Navia et al.³⁸ reported that the whole procedure of dissection, the amplification of the dissected chromosomal material and the labeling of the probe obtained with subsequent FISH could be completed within 72 hours.

Microdissection and tumor cytogenetics

Chromosome rearrangements in leukemic cell populations were studied using micro-FISH by Zhang et al.³⁹ who demonstrated the translocation of ABL to BCR in both classic Ph⁺ CML patients as well as in patients with variant translocations using band-specific probes generated by chromosome microdissection. Deletion of part of the long arm of chromosome 6, one of the most common chromosomal alterations in human B-cell lymphomas was studied by Guan et al.⁴⁰. After the complete dissection of the aberrant chromosome 6, this chromosome was amplified with DOP-PCR and normal metaphases were reverse painted with the probe obtained. They found that the proximal breakpoint was 6q11 in seven of nine cases, with the minimal region of deletion encompassing 6q11 to 6q21. They concluded that, by analogy to other tumor systems, the recurring breakpoints within 6q11 might suggest that a gene important to the genesis or progression of follicular lymphoma could be localized to this band. In tumor cytogenetics micro-FISH is a valuable tool to detect oncogenes amplified in homogeneously staining regions (HSRs) and in double minutes (DMs), as structures like DMs and HSRs can originate from multiple chromosome sites (Guan et al.⁴¹, Su et al.⁴², Ray et al.⁴³ and Guan et al.⁴⁴).

Dissection of 16 HSRs (Guan et al.⁴¹) from nine breast cancer cell lines showed that the most commonly amplified chromosomal band was 17q12 (containing ERBB2). However, regions not containing known oncogenes were also identified, including 13q31 and 20q12-q13.2. Su et al.⁴² have isolated 2 genes from a HSR dissected from an osteosarcoma cell line and mapped these genes to 12q13-q15. In micro-FISH studies of malignant melanoma multiple overexpressed copies of the IGF1R gene were shown to be present in a HSR-bearing cell line (Guan et al.⁴⁵, Zhang et al.⁴⁶ and Zhang et al.⁴⁷).

Another technique now widely used to ascertain which chromosome regions are altered in a malignancy is comparative genomic hybridization (CGH). Colocalization of DNA from tumor and normal cells by dual fluorescent hybridization to normal metaphases generates a profile which identifies regions that are amplified or deleted in the tumor cells. To determine the sensitivity of microdissection in conjunction with CGH Joos et al.⁴⁸ dissected variable numbers of cells from the cell line Colo320. They report that 40 % - 50 % of the imbalances present in the cell line were detected. However, when less than ten nuclei were dissected a high proportion of additional imbalances was found constituting false-positive results. Fujii et al.⁴⁹ used microdissection to isolate infiltrating breast cancer tumor cells from contaminating normal lymphocytes and stromal cells. When analysing microsatellite repeats from chromosome region 6q23->q25.2 they detected a much higher frequency of allelic loss in this chromosome region than had been previously detected in breast cancer. Although micro-FISH is the most direct way to generate region- and band-specific paints and thus to characterize chromosome abnormalities, another method seems to be equally effective for this purpose. This method, described by Jinno et al.⁵⁰, still includes a microchemistry step where microdissected chromosomes are digested with Sau3AI. The chromosome fragments are then ligated to synthesized oligonucleotides using T4 ligase and amplified by PCR. A small part of the PCR product is used then as a FISH probe, the remaining PCR product is usually used for cloning. Deng et al.⁵¹ and Ohta et al.⁵² used this method to characterize unbalanced chromosome translocations, Ohta et al.⁵² and de Albuquerque-Coelho et al.⁵³ disclosed the chromosomal origin of marker chromosomes and Sen et al.⁵⁴ determined the chromosomal content of double minutes.

Quality of the micro-FISH products

For the successful application of micro-FISH certain precautions have to be made. DNA damage due to methanol:acetic acid treatment during fixation should be reduced. Depurination of the chromosomal DNA (Mezzanotte et al.¹⁴) can in part be prevented by using a 9:1 methanol:acetic acid fixation instead of the 3:1 standard formula (Hagag et al.⁵⁵). Furthermore, when cover glass slides holding the metaphases are made, these slides should be washed immediately with distilled water to remove remaining acetic acid. Subsequently, slides should be stored in 95% ethanol at -20 °C until use, and when used for microdissection, a slide should not be used for more than one hour (Hagag et al.⁵⁵). The most important factor for the quality of the DOP-PCR product after microdissection of an eventually constructed library is the effective prevention of contaminant DNA during the microdissection and microcloning steps. Thus, stringent procedures to minimize contamination should be adopted (Kao et al.²⁶). These include: (1) Use of only disposable pipettips, eppendorf tubes, etc; (2) Wearing gloves during all steps of the procedure; (3) Preparing all buffers and reagents in an air-clean hood; (4) Exposing the dissecting needles, micropipets and all buffers and reagents to ultraviolet light (254 nm); (5) Including a control in each experiment treated identical as the dissected sample. If any detectable background PCR product is

found in the control, indicating contamination, the experiment should be discarded. The quality of the DOP-PCR product can be monitored by examining the PCR products after gel electrophoresis and ethidium bromide staining; (6) If the DOP-PCR product is used for microcloning, part of the product should be hybridized *in situ* to metaphase chromosomes to make sure that only the dissected region displays a FISH signal.

Applications of microdissection and micro-FISH in (human) genome analysis

1. Synthesis of region- and band-specific (DOP)-PCR products which 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^(29-33,35-38,56).
2. Synthesis of region- and band-specific (DOP)-PCR products to analyse chromosome aberrations (frequently) occurring in patients with leukaemia^(39,40,57,58).
3. Synthesis of region- and band-specific (DOP)-PCR products to analyse chromosome aberrations occurring in solid tumors e.g. double minutes, homogeneously staining regions and the chromosome aberrations mentioned before^(41-47,59).
4. Isolation of sequences from translocation breakpoints and from small deletions associated with genetic diseases or specific forms of cancer^(39,44,60).
5. Construction of region- or band-specific libraries. From these libraries large numbers of unique sequence microclones can be isolated for molecular genetic and genome studies^(18-24,42-47).
6. The unique clones are valuable for the isolation of YAC clones for contig assembly and for physical mapping and saturation cloning of the dissected region⁶⁰. Furthermore, these clones can be used to isolate candidate genes from cDNA clones to facilitate cloning of disease-related genes⁽⁶¹⁻⁶⁴⁾.
7. Analysis of the molecular structure and organization of specific chromosome landmarks such as centromeres, telomeres, heterochromatin, acrocentric short arms, fragile sites, etc.^(19,65,66).
8. Construction of chromosome region- or band-specific genomic libraries from other than human species, for molecular genetic studies in these species and for comparative studies between species^(2,3,5,6,8,9,11,13,67-70).
9. Microdissection (followed by DOP-PCR) of single cells, specially tumor cells, which can be studied (using CGH) without background contamination from any other cellular or tissue constituents^(48,49).

AIMS OF THIS THESIS

This study was initiated to contribute to the further development of micro-FISH as well as to applicate this technique in routine clinical cytogenetics. The aims of this study were:

1. To develop a fast, reliable and efficient method for microdissection and micro-FISH.
2. To use micro-FISH for the characterization of marker chromosomes that could not (easily) be identified using conventional cytogenetic techniques.
3. To study complex chromosome rearrangements and detect and identify losses and gains of chromosomal material.
4. To study the sensitivity of the developed procedure in detecting loss of chromosomal material.

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Chapter 2

A SIMPLE AND EFFICIENT METHOD FOR MICRODISSECTION AND FISH.

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We developed a simple and efficient method for the dissection of (marker) chromosomes, micronuclei and chromosome regions. Before microdissection, metaphases were overlaid with milli-Q water to rehydrate the chromosomes which made them soft and sticky. The dissected chromosome fragments were dissolved without proteinase-K or topoisomerase treatment and directly amplified using a degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR). The advantages of this method over previously reported methods are: microdissection in this way is very fast; a chromosome, marker, micronucleus or chromosome region is collected as a whole using only one microneedle; the dissected material sticks tightly to the needle without the risk of getting lost; no Sequenase is used in the DOP-PCR reaction which reduced the risk of contamination.

Introduction

Microdissection is introduced in human cytogenetics by Bates et al.¹ who constructed a library for chromosome 2 using many (100 or more) chromosome fragments that were dissected from unbanded and unstained chromosomes. Lüdecke et al.² described an improved procedure for microdissection and microcloning and introduced the PCR technique to amplify the minute amounts of dissected chromosome fragments. This method was improved further by Senger et al.³ who successfully dissected single bands from GTG-banded chromosomes. Micro-FISH, a technique developed by Meltzer et al.⁴, represents at the moment the most straightforward method for the synthesis of chromosome region-specific and chromosome band-specific paints and thus for the characterization of chromosome aberrations. In this technique four important steps can be distinguished: (1) the physical dissection of (part of) a chromosome or a marker chromosome, (2) incubation of the dissected material with proteinase-K or topoisomerase to relax the supercoiled DNA of the metaphase chromosome(part)s, (3) degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) to amplify the dissected material and (4) labeling of the PCR product with biotin or digoxigenin followed by fluorescence in situ hybridization with the probe obtained. In this study we describe an improved and simplified method for microdissection, dissolvment of the dissected material and DOP-PCR with less risk of contamination.

Material and methods

Preparation of metaphase chromosomes

Chromosomes were prepared from peripheral blood lymphocytes using the synchronization method of Dutrillaux and Viegas-Pequignot⁵ with only minor modifications. Chromosome spreads for microdissection were made according to routine procedures on 45x65 mm coverslips. The slides were rinsed in distilled water and stored in 98% ethanol at -20 °C. GTG-banding with trypsin-Giemsa was performed prior to microdissection.

Microdissection of banded chromosomes

Microdissection was performed with glass microneedles controlled by a Narishige micromanipulator (MO-202). To avoid contamination with extraneous DNA microneedles, microcentrifuge tubes (including the collection drop) and pipettips were treated with ultraviolet light for 30 min. Before microdissection of a (marker) chromosome we looked for a metaphase with well spread chromosomes that was covered with 2 µl milli-Q water. Due to the rehydration the chromosomes became sticky and could be collected as a whole using only one microneedle. To collect a chromosome band or region, a slightly different procedure was followed. The chromosome bands proximal and distal of the band or region of interest were dissected when the chromosomes were still dry; subsequently a new microneedle was installed, the metaphase was covered with 2 µl milli-Q water and the chromosome band or region was dissected. In the experiments five chromosome bands or regions were collected in this way with the

chromosome (part) glued to the needle tip and transferred to a 20 µl collection drop (containing 2.5mM MgCl₂, 50mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatine, 0.1% (v/v) Brij-35 (Sigma)) in a 0.5 ml microcentrifuge tube. A fresh microneedle was used for each dissection.

Amplification of chromosomal DNA

After sufficient chromosomal material was collected, the samples were cycled for 20 cycles of 30 °C for 1 min and 50 °C for 1 min to dissolve the dissected chromosome (part)s. Subsequently, 30 µl PCR buffer containing 100 µM of each dNTP, 2,5mM MgCl₂, 50mM KCl, 10 mM Tris-HCl pH 8.3, 5 ng acetylated BSA, 4 µM universal primer and 0.5 U super Taq DNA polymerase (HT Biotechnology) was added to each sample. The universal primer 5'CCGACTCGAGNNNNNNATGTGG 3' was used as suggested by Telenius et al.⁶ For initial denaturation the mixture was heated to 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 at 94 °C, 1 min at 56 °C, 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 microdissected DNA and a positive control with 25 pg total human DNA. PCR reactions were performed in a Biometra personal cycler with a heated lid. PCR products were stained with ethidium bromide and analysed for yield and probe size (200-900 base pairs) on an agarose gel. Experiments were proceeded only if DNA synthesis was not apparent in the negative control. The PCR product was precipitated and purified with NH₄Ac (4 M) and isopropanol, washed with ethanol (70%) and dissolved in 60 µl T0,1E buffer (10 mM TRIS-HCl/0,1 mM Na₂EDTA, pH 8.0).

Fluorescence in situ hybridization (FISH).

PCR products were labeled by nick-translation with biotin-14-dATP (BioNick™ labeling system (Gibco BRL)). Metaphase spreads for FISH were prepared from PHA-stimulated lymphocytes using standard methanol: acetic acid (3:1) fixation. *In situ* hybridization was performed following the protocol by Guan et al.⁷ with only minor modifications. Briefly, for hybridization, 200 ng probe was used in 10 µl hybridization mixture containing 50% formamide, 2xSSC, 10% dextran sulphate, 50mM phosphate and 20 µg human Cot 1 DNA (BRL). Probe and Cot 1 DNA were denaturated at 70 °C for 5 min and reannealed at 37 °C for 90 min. Slides were denaturated in 70% formamide, 2xSSC (pH 7.0) at 70 °C for 3 min, dehydrated and hybridized with probe at 37 °C overnight in a moist chamber. Slides were then washed four times in 2xSSC (pH7.0) at 45 °C for 5 min and one wash in PN buffer (0.1 M sodium phosphate, 0.1% (v/v) Nonidet P-40, pH 8.0) at 45 °C for 10 min. The hybridization signal was detected by three layers of fluorescein-avidin DCS (Vector) amplified by two layers of biotinylated anti-avidin D (Vector), followed by two washes with 4xSSC, 0.5% Tween and one wash with PBS. Slides were dehydrated and counterstained with DAPI/PI in antifade solution. Analysis was carried out using a Zeiss Axiophot microscope and photographs taken with Scotch chrome 640 ASA colour slide film.

Fig.1: 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 and d:** Dissection of chromosome 1. **e and f:** Dissection of the q-arm of chromosome 2. **g:** Removal of centromere 7 and band 7q13. **h:** Dissection of chromosome region 7q11-q12. **i and k:** Dissection of a micronucleus.

Results and discussion

In the procedure that is nowadays used to prepare metaphase chromosomes for microdissection, special attention is paid to prevent depurination of the DNA (Mezzanotte et al.⁸). When preparing slides for microdissection they are washed with distilled water to remove acetic acid and then immediately stored in 98% ethanol at -20 °C. Due to fixation procedure and storage in ethanol chromosomes are dehydrated and become hard (Claussen et al.⁹). This hampers the microdissection, especially of whole chromosomes and chromosome regions. An additional problem that frequently occurs is the repulsion of the dissected fragments from the needle. Bussey¹⁰ described it fondly as the "Mexican jumping bean" phenomenon. The procedure reported here overcomes these problems. Before microdissection of a whole chromosome, a marker chromosome or a micronucleus, the microneedle is brought into the field within a few micrometers of a well spread metaphase (fig. 1a). This metaphase is covered with 2 µl milli-Q water (fig. 1b), the microneedle is then moved down on the slide and the (marker) chromosome of interest is detached entirely from the slide using just one needle (fig. 1d). For the dissection of a chromosome region or a chromosome band a slightly different procedure has to be followed. First, the chromosome region or chromosome band of interest is isolated from the rest of the chromosome by dissecting the chromosome parts proximal and distal of the region or band of interest (fig. 1e and 1g). This manipulation is performed when the chromosome is still dry and hard. Next, a new microneedle is installed, positioned slightly above the metaphase which is then covered with 2 µl milli-Q water. Finally, the chromosome region or band is dissected using only one needle (fig. 1f and 1h). Beside the fact that this procedure of microdissection is very fast, dissection of a chromosome is completed within a minute, a second advantage is that the microdissected chromosome part sticks tightly to the needle tip (even after the tip is removed out of the water drop) without the risk of losing the dissected fragment when transferring it to the microcentrifuge tube containing the collection drop. The second step in the micro-FISH procedure is treatment of the dissected material to dissolve chromosomal DNA. Proteinase-K treatment (Jinno et al.¹¹; Deng et al.¹²; Meltzer et al.⁴) is used to digest the proteins that are associated with the DNA of mitotic chromosomes and also pretreatment with topoisomerase I is advised (Guan et al.¹³) to enhance the accessibility of the dissected DNA for primers and polymerase during the following PCR. In our experiments the samples were cycled for 20 cycles of 30 °C for 1 min and 50 °C for 1 min, without proteinase-K or topoisomerase treatment, which led to a yield of DNA after DOP-PCR comparable with the proteinase-K method we used previously (Engelen et al.^{14,15,16}). The most crucial step in micro-FISH is, without any doubt, the amplification of the dissected material using DOP-PCR. Although various protocols for DOP-PCR exist, most authors prefer at the moment the method developed by Guan et al.¹³. In the first 8 cycles of this method, when the annealing temperature is low, T7 DNA polymerase (Sequenase) is used. However, Sequenase is heat inactivated at the denaturation temperature of 94 °C and consequently active Sequenase has to be added 8 times. For this reason, these steps are a prime target for contamination. In the method described

here the use of Sequenase is omitted, which reduces the risk of extraneous contamination considerably.

For standard applications a primer concentration between 0.1 and 1 μM is recommended (Rolfs et al.¹⁷) and rarely the primers are completely used up during the reaction. Nevertheless, the primers have to compete with the accumulating product in finding the target sequences, which could become a limiting factor for the reaction, certainly at the late cycles. We decided to use a high primer concentration (4 μM) in our experiments, as Czerny¹⁸ showed that primer limitation can make a critical contribution to the attenuation of amplification rates observed for late cycles of PCR. As an example, fig. 2a shows the results of micro-FISH of the q-arm of chromosome 2, dissected in fig. 1e and 1f. Furthermore, fig. 2e shows that a micronucleus from a patient with ICF syndrome (Smeets et al.¹⁹) contains DNA derived from chromosome 1, and fig. 2c shows a chromosome 7q12 band-specific paint.

In conclusion, the modified microdissection method described here enabled fast and reliable dissection of chromosomes and regions of chromosomes. Furthermore, relaxation of the dissected material was achieved without extra additives, an efficient DOP-PCR reaction mix was composed and reduction of the risk of extraneous contamination was reached by omitting Sequenase addition.

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Fig.2: Partial metaphase after reverse painting to a normal metaphase with biotinylated PCR product:
a: derived from the q-arm of chromosome 2. **c:** derived from chromosome region 7q11-q12. **e:**
 derived from the micronucleus of an ICF patient. **b, d and f:** DA/DAPI counterstaining of a, c and e.

MARKER CHROMOSOME IDENTIFICATION BY MICRO-FISH.

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Micro-FISH was used to elucidate the chromosomal origin of marker chromosomes in three patients. Ten copies of marker chromosomes were collected with microneedles from GTG-banded metaphases, transferred to a collecting drop and amplified by means of DOP-PCR. The PCR products were labeled with biotin-14-dATP and used as FISH probes for hybridization to normal metaphase chromosomes and to metaphase chromosomes of the patients (reverse painting). With the generation of chromosome region-specific painting probes by PCR amplification of microdissected DNA and subsequent FISH it was possible to identify the marker chromosomes in all patients. One marker appeared to be derived from the centromere region of the X-chromosome and the proximal third of the long arm, one from the centromere region of chromosome 17 and one marker chromosome was identified as an isochromosome 18p.

Introduction.

A marker chromosome (mar) is a structurally abnormal chromosome in which no part can be identified (ISCN 1995). They constitute a heterogeneous group of supernumerary chromosomes which often are not identifiable by cytogenetic banding techniques, mainly due to their small size and the lack of a distinct banding pattern. Constitutional markers are found with a frequency of 0.24/1000 in lifeborn individuals (Buckton et al.²) and 0.4 - 1.5/1000 at amniocentesis (Hook and Cross³, Sachs et al.⁴, Warburton et al.⁵). The incidence of marker chromosomes in mentally retarded patients is reported to be 3.27/1000 (Buckton et al.²). NOR staining and distamycin A/DAPI staining have shown that over 80% of the marker chromosomes originate from the pericentromeric regions of the acrocentric chromosomes (Maraschio et al.⁶). Approximately 50% of these markers are DA/DAPI positive and therefore originate from the centromere region of chromosome 15 (Maraschio et al.⁶, Blennow et al.⁷, Plattner et al.⁸). Beside these conventional staining techniques, more recently, fluorescence *in situ* hybridization, often preceded by chromosome flow sorting, microcloning or degenerate oligonucleotide primed-PCR (DOP-PCR) has been used to identify marker chromosomes (Rauch et al.⁹, Plattner et al.¹⁰, Blennow et al.⁷ and Crolla et al.¹¹). However, the most straightforward approach for identification is, without any doubt, the physical dissection of marker chromosomes followed by DOP-PCR, labeling of the PCR products and subsequently, reverse painting (micro-FISH). In this study we describe the characterization of two unknown marker chromosomes and we confirm the nature of one cytogenetically identified marker chromosome by FISH with DOP-PCR products derived from ten copies of microdissected marker chromosomes.

Material and methods

Preparation of metaphase chromosomes

PHA-stimulated peripheral blood lymphocytes were cultured and harvested following the standard procedures. Routinely fixed (methanol:acetic acid 3:1) cell suspensions stored at -20 °C for 3 (patient A), 5 (patient B) and 10 months (patient C) were used to prepare metaphase spreads on 45x65 mm coverslips. The slides were rinsed in water and stored in 98% ethanol at -20 °C until the day of microdissection. GTG-banding was performed prior to microdissection.

Microdissection and amplification of chromosomal DNA

From ten different metaphases marker chromosomes were collected by microdissection using glass microneedles controlled by a Narishige micromanipulator (MO-202). Before use, microneedles and microcentrifuge tubes were treated with UV light for 30 min. The dissected marker chromosomes were transferred to a 20 µl collection drop (containing 250 µg/ml proteinase-K) in a 0.5 ml microcentrifuge tube. A fresh microneedle was used for each marker chromosome. Before DOP-PCR, the collection drop containing ten copies of the markers, was incubated at 37 °C for 30 min (proteinase-K treatment) and at 90 °C for 10 min (proteinase-K inactivation). DOP-

PCR reactions were performed following the protocol of Guan et al.¹² in a Biometra personal cycler with a heated lid. PCR products were analysed for yield and probe size (200-800 bp) on an agarose gel after staining with ethidium bromide. The PCR product was precipitated and purified with NH₄Ac (4 M) and isopropanol, washed with ethanol (70%) and dissolved in 50 µl T0,1E buffer (10 mM TRIS-HCl/0,1 mM Na₂EDTA, pH 8.0).

Fluorescence *in situ* hybridization (FISH).

PCR products were labeled by nick-translation with biotin-14-dATP. Metaphase spreads for FISH were prepared from PHA-stimulated lymphocytes using the standard 3:1 (v/v) methanol: acetic acid fixation protocol. *In situ* hybridization was performed following the protocol of Pinkel et al.¹³ with only minor modifications. For hybridization, 200 ng probe was used in 10 µl hybridization mixture containing 50% formamide, 2xSSC, 10% dextranesulphate, 50mM phosphate, 3 µg human Cot 1 DNA and 10 µg salmon sperm DNA. Whole chromosome painting with chromosome specific paint was performed according to the manufacturer's specification (Oncor). Analysis were carried out using a conventional Zeiss epifluorescent Axiophot microscope and photographs taken with Scotch chrome 640 ASA colour slide film.

Clinical reports and cytogenetics

Patient A was an 11-year-old girl referred for chromosome investigation because of growth retardation (height=126cm, <P3). Clinical features suggestive for Turner syndrome were low posterior hairline, shield chest, widely spaced nipples, numerous naevi and dysplastic toe nails. The child was not mentally retarded and displayed no other dysmorphic features.

Analysis of GTG-banded chromosomes of the patient revealed that only one X-chromosome was present in 40% of the cells while 60% had beside a normal X-chromosome a marker chromosome (Fig. 1A). The karyotype formula was mos 45,X/46,X,+mar. Conventional Giemsa staining and C-banding showed that the marker chromosome was not a ring chromosome and had a centromere (Fig. 1B). The size of the marker was similar with that of the G-group chromosomes. NOR-staining showed that there were no satellites. The karyotypes of the parents were normal.

Patient B was a 42-year-old female with moderate mental retardation, small stature, asthenic body habitus and microcephaly (HC 50,5 cm, <P3). Facial features included oval facial shape, narrow forehead, up-slanting and small palpebral fissures (PFL 2,5 cm bilaterally, <-2SD), hypotelorism (ICD 2,5 cm, <-2SD), a small nose and slightly malformed ears.

In all cells of the patient an extra isochromosome (Fig. 1C) was present, the karyotype being 47,XX,+mar. The GTG banding pattern suggested an isochromosome 18p. Both parents had normal karyotypes.

Patient C was a 4-year-old girl referred for cytogenetic examination because of growth retardation, delayed speech development and muscle weakness, predominantly of the distal lower limbs. Chromosome analysis showed a normal 46,XX karyotype in 12% of the cells, a 47,XX,+mar karyotype in 40% and a 48,XX, +mar,+mar karyotype in

48% of the cells. In GTG-banded metaphases all marker chromosomes had the same appearance and size, which was about half that of chromosome 21 (Fig. 1D). Conventional Giemsa staining revealed that we were not dealing with a ring chromosome. NOR staining showed that the markers had no satellites and C-banding showed that a centromere was present (Fig. 1E). Both parents had normal karyotypes.

Fig. 1A: GTG-banded marker chromosome of patient A. 1B: C-banded marker chromosome of patient A. 1C: GTG-banded marker chromosome of patient B. 1D: GTG-banded marker chromosome of patient C. 1E: C-banded marker chromosome of patient C. Arrows point to the marker chromosome.

Results

Patient A: FISH on normal female metaphase chromosomes with the DOP-PCR product showed signals in the centromeres of two chromosomes of the C-group and a signal extending in the long arm (Fig. 2A). After analysis of the DAPI counterstained chromosomes it became clear that the chromosomes involved were the X-chromosomes. FISH on metaphase chromosomes of the patient with the DOP-PCR product again showed a strong signal in the centromere of the X-chromosome and a (weaker) signal on the proximal third of the q-arm. On the marker chromosome a strong signal was present in what is presumably the centromere, however the euchromatic region of the marker was less intensively stained (Fig. 2B). Chromosome painting with a whole chromosome paint specific for the X-chromosome showed that the marker chromosome consisted completely of X-chromosomal material (Fig. 2D).

Patient B: FISH with the DOP-PCR probe on normal female metaphase chromosomes (Fig. 2E) and on metaphase chromosomes of the patient showed that the short arm of two chromosomes of the E-group were completely stained, together with the marker chromosome in the patient (Fig. 2F). DAPI counterstaining showed that chromosome 18 was indeed involved (Fig. 2G). FISH with a whole chromosome paint specific for chromosome 18 painted both chromosomes 18 and the isochromosome (Fig. 2H) thus confirming our conclusion. The karyotype of this patient is 47,XX,i(18p).

Patient C: FISH with the DOP-PCR product on normal female chromosomes showed fluorescent spots in the centromeres of two chromosomes belonging to the E-group and some fluorescence on the short arm of these chromosomes (Fig. 2J). FISH with DOP-PCR product on metaphases of the patient also showed a signal in the centromere of two chromosomes of the E-group and the marker chromosomes, which were completely painted (Fig. 2K). Analysis of the DAPI counterstained chromosomes (Fig. 2L) led to the conclusion that the marker chromosomes originated from chromosome 17. FISH with a whole chromosome paint specific for chromosome 17 confirmed this conclusion (Fig. 2M).

Discussion

In recent years several FISH methods have been developed and used for the identification of marker chromosomes. Firstly, FISH with chromosome specific probes (Schwartz et al.¹⁴, Callen et al.¹⁵, Rauch et al.⁹) provided a tool for identification. Stepwise hybridization with alpha-satellite probes under multiple stringency conditions was used by Plattner et al.^{8,10} to identify 20 small DA/DAPI negative ring and 7 DA/DAPI positive marker chromosomes. Secondly, fluorescence activated cell sorting and DOP-PCR enabled Carter et al.¹⁶ and Blennow et al.⁷ to construct marker specific libraries. However, chromosome size (Young¹⁷) and marker chromosome heterogeneity (Cram et al.¹⁸) are limiting factors in chromosome sorting. Thirdly, Senger et al.¹⁹, Lengauer et al.²⁰ and Hirota et al.²¹ used (laser)-microdissection in combination with microcloning for chromosome region-specific cloning and probe generation. Lastly, biotin-11-dUTP labeled PCR products of microdissected chromosomes were directly used for reverse painting (Meltzer et al.²², Deng et al.²³, Guan et al.^{24,25}). Deng et al.²³ used Sau3A1 digests of 5-10 chromosome fragments for linker-primer PCR. Meltzer et al.²² who initially worked with 25-50 microdissected copies, were able to reduce the copy number to 1 (Guan et al.²⁴) by treatment of the DNA with topoisomerase I before DOP-PCR. The latter approach could be particularly suitable in tumor cytogenetics where material is very limited. Thangavelu et al.²⁶, Viersbach et al.²⁷ and Sun et al.²⁸ used micro-FISH for the identification of marker chromosomes. However, the DOP-PCR probes they generated showed cross-hybridization to the satellites of chromosomes of the D- and G-group. Highly repetitive beta satellite DNA present in the short arms of the acrocentric chromosomes (Waye and Willard²⁹) might be responsible for this cross-hybridization. They came to a final identification of the marker chromosomes using specific probes for the chromosomes involved.

Our study has demonstrated that microdissection of marker chromosomes followed by DOP-PCR can be used to generate probes that hybridize all over the marker chromosome without systematic cross-hybridization to non-targeted chromosomes. Micro-FISH is a reliable method to identify marker chromosomes, because they can easily be recognized due to the combination of reverse painting and DA/DAPI counterstaining. Patient A has a marker that is derived from the X-chromosome, containing the centromere and the region Xq11 to Xq21. Individuals with a cell line containing a small mar(X) chromosome often have mental retardation and dysmorphic features uncharacteristic for Turner syndrome (Wolff et al.³⁰). The abnormalities seen in these patients may be due to failure of X-chromosome inactivation, resulting in functional disomy of pericentromeric sequences (Van Dyke et al.³¹). Our patient has a Turner syndrome phenotype without influence of the marker chromosome on her phenotype, probable due to the presence of the X-inactivation center in the marker and non-random inactivation of the marker. After micro-FISH a strong signal was present in presumably the centromere of the marker chromosome and a weaker signal on the euchromatine derived from the q-arm. Perhaps the highly repetitive DNA sequences that exist in centromere regions (Waye and Willard^{29,32}) are responsible for this difference in signal intensity, although it was not present in both other marker chromosomes. In patient B a 47,XX,i(18p) karyotype was found by cytogenetic analysis and

confirmed with micro-FISH. Her clinical features correspond well with the isochromosome 18p syndrome (Callen et al.³³). Patient C has a mosaic 46,XX/47,XX,+mar/48,XX,+mar,+mar karyotype whose marker chromosome is derived from the centromere region of chromosome 17. Although Hagag and Viola³⁴ and several other authors mentioned that acetic acid fixation of the cells causes degradation of the DNA by depurination and recommend a short fixation time and ethanol treatment of cells, our results with a fixed cell suspension kept at -20 °C for three years were quite satisfactory (unpublished data).

The advantages of micro-FISH above the methods mentioned before are that it is less time consuming than the microcloning procedures (a marker chromosome can be identified in three days) and in comparison to chromosome sorting, the size of the marker is not a limiting factor. Furthermore, only a small number of dissected chromosomes is needed and at least up to three years old fixed cell suspensions can be used making retrospective analysis possible. Finally, marker identification with centromere probes or paints discloses the origin of the marker but not the chromosome region involved. Since the collection of banded chromosomes by microdissection can be precisely controlled, libraries for screening can be made, beside chromosome region-specific and chromosome band-specific paints. Thus, micro-FISH is applicable not only for cytogenetically unidentifiable marker chromosomes, but also for the diagnosis of numerical and structural chromosome abnormalities and genome analysis.

Fig. 2A: FISH with the DOP-PCR probe of patient A on a metaphase of a normal woman. **2B:** DOP-PCR probe of patient A hybridized to a metaphase of patient A. **2C:** DA/DAPI counterstaining of 2b. **2D:** X-chromosome specific paint hybridized to a metaphase of patient A. **2E:** FISH with the DOP-PCR probe of patient B on a metaphase of a normal woman. **2F:** DOP-PCR probe of patient B hybridized to a metaphase of patient B. **2G:** DA/DAPI counterstaining of 2f. **2H:** Chromosome 18 specific paint hybridized to a metaphase of patient B. **2J:** FISH with the DOP-PCR probe of patient C on a metaphase of a normal woman. **2K:** DOP-PCR probe of patient C hybridized to a metaphase of patient C. **2L:** DA/DAPI counterstaining of 2 K. **2M:** Chromosome 17 specific paint hybridized to a metaphase of patient C. Large arrows point to the marker chromosomes; small arrows point to the normal chromosomes from which the marker chromosomes originate.

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**DISCLOSURE OF FIVE BREAKPOINTS IN A COMPLEX CHROMOSOME
REARRANGEMENT (CCR) BY MICRODISSECTION AND FISH.**

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Microdissection and fluorescence in situ hybridization (FISH) were used to elucidate the nature of a complex chromosome translocation, after GTG-banding failed in the complete characterization of the structural rearrangement between chromosomes 6 and 12. These chromosomes were painted with chromosome specific paints and one of the chromosome regions involved in the translocation was isolated by microdissection. Ten copies of the microdissected region were collected with microneedles from GTG banded metaphases, transferred to a collecting drop and amplified by means of DOP-PCR. The PCR product was labeled with biotin-14-dATP and used as a FISH probe for hybridization to normal metaphase chromosomes and metaphase chromosomes of the patient (micro-FISH). FISH with this chromosome region-specific painting probe and with chromosome band-specific probes enabled the characterization of a complex chromosome rearrangement with five breakpoints in two chromosomes. This resulted in the following karyotype: 46,XY,t(6;12)(6pter->6q12::12q24.1->12qter;12pter->12q13.3::6q16.2->6q26::12q13.3->12q24.1::6q12->6q16.2::6q26->6qter).

Introduction

A complex chromosome rearrangement (CCR) has been defined as an interchange comprising more than two chromosome breaks and reciprocal exchange of segments (Pai et al.¹). Since interpretation of a CCR is often difficult when only standard GTG-banding techniques are used, FISH with whole chromosome paints, developed by Pinkel et al.² and Lichter et al.³ is frequently used for characterization of a CCR (Verma et al.⁴; Batista et al.^{5,6}; Wang et al.⁷). However, in the case reported here, the application of GTG-banding and whole chromosome painting still did not characterize the CCR completely. Micro-FISH is the physical dissection of (GTG)-banded chromosomes followed by (DOP)-PCR and subsequent FISH with the probe obtained. This method, developed by Meltzer et al.⁸ and Deng et al.⁹ is successfully used to generate whole chromosome painting probes (Guan et al.¹⁰) region-specific probes (Guan et al.^{11,12}) and band-specific probes (Hirota et al.¹³; Guan et al.¹⁴). In clinical cytogenetics micro-FISH is used to identify marker chromosomes (Thangavelu et al.¹⁵; Viersbach et al.¹⁶; Sun et al.¹⁷; Engelen et al.¹⁸) and unidentifiable chromosome abnormalities (Ohta et al.¹⁹).

We describe the use of micro-FISH in a case in which chromosomes 6 and 12 are involved in a CCR with five breakpoints (Fig. 1). Whole chromosome painting and micro-FISH were very beneficial in identifying the rearranged segments. Additionally, FISH with centromere-specific probes and band-specific probes was essential for the complete characterization of the CCR.

Material and methods

Clinical report

The patient was an 18-year old male whose chromosomes were re-examined because of a strong suspicion of an unbalanced karyotype. Cytogenetic analysis at three years of age had revealed a reciprocal translocation (6;12)(q12;q24). At that time he was referred because of moderate delay in psychomotor development. Now, at the age of 18 years he is a moderate mentally retarded male with normal phenotype. The parents of the patient had normal karyotypes.

Cytogenetics

Chromosomes were prepared from peripheral blood lymphocyte cultures using a modification of the synchronization method of Dutrillaux and Viegas-Pequignot²⁰ by treatment overnight with thymidine, followed by incubation with 5-BrdU for 6 hours and ethidium bromide for one and a half hours before harvest. High-resolution banding was performed by treatment with trypsin followed by staining with Giemsa to obtain a GTG-banded pattern.

Fig. 1: Partial karyotype presenting normal GTG-banded chromosomes 6 and 12 (left) and derivative chromosomes (right). Arrows indicate breakpoints, asterisks indicate points of reunion.

Microdissection and amplification of chromosomal DNA

For microdissection, routinely fixed (methanol:acetic acid 3:1) cell suspensions stored at -20°C were used to prepare metaphase spreads on coverslips. The slides were rinsed in water and stored in 98% ethanol at -20°C . Dissection of the distal region of the der(12) chromosome (fig. 2) was performed with glass microneedles controlled by a Narishige micromanipulator (MO-202). Before use, the needles and centrifuge tubes were treated with UV light for 30 min. The dissected chromosome parts were transferred to a 20 μl collection drop (containing 250 $\mu\text{g/ml}$ proteinase-K) in a 0.5 ml centrifuge tube. A fresh microneedle was used for each dissection. Before DOP-PCR, the collection drop, containing ten copies of the dissected region, was incubated at 37°C for 30 min (proteinase-K treatment) and at 90°C for 10 min (proteinase-K inactivation). PCR reactions were performed following the protocol of Guan et al.¹¹ in a Biometra Personal Cycler with a heated lid. PCR products were analysed for yield and probe size (200-800 bp) on an agarose gel and stained with ethidium bromide. The PCR product was precipitated and purified with NH_4Ac (4 M) and isopropanol, washed with ethanol (70%) and dissolved in 50 μl T0,1E buffer (10 mM TRIS-HCl/0,1 mM Na_2EDTA , pH 8.0).

Fig. 2: Der(12) chromosome before, during and after microdissection. The arrow points to the chromosome portion that is used for DOP-PCR.

Fluorescence in situ hybridization (FISH)

In this analysis the following chromosome specific probes were used:

Chromosome region	Name of probe	Insert (Kb)	Reference
Cen. 6	p308	3.00	Jabs et al. ²¹
Cen. 12	pa12H8	1.35	Looijenga et al. ²²
6q26	cCI6-60	40	Nakamura Y, Personal Comm.
6q27	cCI6-107	40	Nakamura Y, Personal Comm.

Paints specific for chromosomes 6 and 12 and labeled with biotin-11-dUTP were purchased (Oncor) and painting was performed according to the manufacturer's specifications. FISH with the DOP-PCR product was performed following the protocol of Pinkel et al.²³ with only minor modifications. The centromere-specific and band-specific probes were labeled by nick translation with biotin-11-dATP. For FISH with these probes the protocol of Lichter et al.²⁴ was followed. The slides were examined with a Zeiss Axiophot microscope and photographed using Scotchchrome 640 ASA colour slide film.

Results

Cytogenetic re-examination of GTG-banded metaphases revealed a complex chromosomal rearrangement of the chromosomes 6 and 12 in which at least four regions were involved (fig. 1). Painting with a chromosome 6 and a chromosome 12 specific paint confirmed this conclusion (fig. 3A and 3B). However, GTG-banding combined with chromosome painting was not sufficient to characterize the distal region of the der(12) chromosome completely. Ten copies of the distal region of the der(12) chromosome were collected by microdissection (fig. 2) and the probe that was generated by DOP-PCR was hybridized to normal metaphase chromosomes and to metaphase chromosomes of the patient. Normal metaphases showed a signal in the region 6q12 to 6q16.2 and a second signal in the distal region of chromosome 6 (fig. 3C). On metaphase chromosomes of the patient the same fluorescence pattern as described above was seen on the normal chromosome 6 and, as expected, a fluorescent signal was seen at the distal end of the der(12) chromosome (fig. 3D). FISH with probe cCI6-60 specific for chromosome band 6q26 showed that the probe hybridized at the distal end of the normal chromosome 6. In the der(12) chromosome the corresponding fluorescent spot was detected in the middle of the long arm (fig. 3E). Probe cCI6-107 specific for chromosome band 6q27 hybridized at the distal end of the normal chromosome 6 and at the distal end of the der(12) chromosome (fig. 3F).

Fig. 3: (A) Metaphase chromosomes of the patient after FISH with a chromosome 6 specific paint. (B) Metaphase chromosomes of the patient after FISH with a chromosome 12 specific paint. (C) Metaphase of a normal male after FISH with the DOP-PCR probe generated from the der(12). Arrows point to the signals on the q arm of chromosomes 6. (D) Metaphase of the patient after FISH with the DOP-PCR probe generated from the der(12) chromosome. Signals are on the q arm of chromosome 6 and on the q arm of the der(12) chromosome. (E) Metaphase of the patient after FISH with centromere probe p308 (6) and probe pa12H8 (12) together with probe cCI6-60 specific for chromosome band 6q26. Arrows point to the signals on the q arm of chromosome 6 and the q arm of the der(12) chromosome. (F) Same as fig. 3E with probe cCI6-107 specific for chromosome band 6q27.

Discussion

High-resolution GTG-banding is usually sufficient to identify chromosome translocations. However, for the clarification of complex chromosome rearrangements, FISH with chromosome painting probes is nowadays almost indispensable. At the moment painting probes are available for all chromosomes and their use has led to a reinterpretation of the karyotype in at least one other previously reported case with a CCR (Batista et al.⁶). In the present case, the combined data of GTG-banding and chromosome painting still left some doubts about the structure of the der(12) chromosome. Microdissection has provided a means for characterization of chromosome bands and chromosome regions. Several laboratories have previously utilized microdissection of GTG-banded human metaphase chromosomes to generate region-specific libraries. The first studies used restriction endonuclease digestion and DNA ligation steps (Johnson et al.²⁵; Lüdecke et al.²⁶; Senger et al.²⁷; Davis et al.²⁸; Lengauer et al.²⁹). Later, Meltzer et al.⁸ developed a technique that eliminated the microchemistry procedure by making use of a DOP-PCR and cloning procedure. Recently, Guan et al.^{10,11,12} used DOP-PCR probes generated from microdissected GTG-banded chromosomes for chromosome (region) painting.

In our case, whole chromosome painting disclosed that at least 6 regions (and 4 breakpoints) were involved in this complex rearrangement. However, micro-FISH of the distal region of the der(12) chromosome showed that this region originates from two different segments of chromosome 6 namely 6q12-6q16.2 together with a distal region of 6q thus disclosing a fifth breakpoint. FISH with probes specific for chromosome bands 6q26 and 6q27 further characterized this fifth breakpoint in chromosome 6 between these bands. The patient's karyotype is: 46,XY,t(6;12)(6pter->6q12::12q24.1->12qter;12pter->12q13.3::6q16.2->6q26::12q13.3->12q24.1::6q12->6q16.2::6q26->6qter). As loss or gain of chromosomal material was not detected, the mental retardation of our patient can best be explained by a small submicroscopic deletion or by disruption of a gene or regulatory sequence caused by one (or more) of the five breakpoints. This assumption is supported by the increased risk on mental retardation and phenotypic abnormalities in carriers of apparently balanced *de novo* translocations (Warburton et al.³⁰) or *de novo* CCRs (Kousseff et al.³¹; Kausch et al.³²) when compared with familial cases.

In summary, the use of chromosome painting, micro-FISH and FISH with band-specific probes showed a CCR of the chromosomes 6 and 12 in which five breakpoints were involved. The application of these techniques provides a powerful tool in the characterization of constitutional and acquired complex structural aberrations. Furthermore, the results of our study show that the generated DOP-PCR probe contains DNA sequences that span a breakpoint. Microdissection in combination with DOP-PCR and subsequent cloning will enable the isolation of sequences from translocation breakpoints associated with genetic diseases or specific forms of cancer that can be used in metaphase and interphase cytogenetics and in genome analysis.

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APPLICATION OF MICRO-FISH TO DELINEATE DELETIONS.

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Microdissection in combination with fluorescence in situ hybridization (micro-FISH) is used to visualize deletions in rearranged chromosomes and in a de novo translocation. In each experiment five copies of a structurally aberrant chromosome or of the two chromosomes involved in a de novo translocation were isolated by microdissection and amplified using DOP-PCR. The PCR products were then used as probes for fluorescence in situ hybridization (FISH) to metaphase chromosomes of the patients. After reverse chromosome painting, the structurally aberrant chromosomes were completely painted and the region deleted in the aberrant chromosomes was visible in the normal chromosomes. The smallest deletion that could be demonstrated this way was a microdeletion of approximately 6×10^6 bp which is frequently reported in Angelman and Prader-Willi syndromes.

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 (Lichter et al.¹; Pinkel et al.²). However, these techniques have a disadvantage in that probes and chromosome paints to be used must be tested empirically when cytogenetically unidentifiable chromosome abnormalities, such as marker chromosomes and unbalanced *de novo* translocations are involved. Meltzer et al.³ and Deng et al.⁴ 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 overcomes this limitation. Micro-FISH has been applied in clinical cytogenetics to identify marker chromosomes (Thangavelu et al.⁵; Viersbach et al.⁶; Sun et al.⁷; Muller-Navia et al.⁸; Engelen et al.⁹), unbalanced translocations (Ohta et al.¹⁰) and complex chromosome rearrangements (Zhang et al.¹¹; Engelen et al.¹²). In this report, the results of using microdissection combined with DOP-PCR and reverse chromosome painting (Carter et al.¹³) are presented. Application of micro-FISH made it possible to demonstrate the loss of chromosomal material in a *de novo* translocation and to characterize deletions in aberrant chromosomes more precisely.

Material and methods

Cases

Case 1 is a newborn girl referred for cytogenetic examination because of blepharophimosis, microphthalmia, low implanted ears, enlarged ventricles, long fingers and clinodactylia of the left digits III and IV.

Case 2 is an obese 3 year-old boy with psychomotor retardation, strabismus, pes equinovarus and facial anomalies.

Case 3, a girl, was referred at the age of 8 mo for diagnostic examination because of developmental retardation, generalised hypotonia, protruding tongue and at times inexpedient motoric anxiety.

Preparation of metaphase chromosomes

Chromosomes were prepared from peripheral blood lymphocytes using the synchronization method of Dutrillaux and Viegas-Pequignot¹⁴ with minor modifications. For microdissection routinely fixed (methanol:acetic acid 3:1) cell suspensions stored at -20 °C were used to prepare metaphase spreads on 45x65 mm coverslips. The slides were rinsed in water and stored in 98% ethanol at -20 °C. GTG-banding with trypsin-Giemsa was performed prior to microdissection.

Figure 1: Case 1: G-banded normal and deleted chromosome 3 with corresponding ideograms. **Case 2:** G-banded normal chromosomes 14 and 18 and derivative chromosomes 14 and 18 with corresponding ideograms. **Case 3:** G-banded translocation chromosome and deleted chromosome 15 with corresponding ideograms. Arrows indicate the breakpoints.

Microdissection and amplification of chromosomal DNA

Microdissection was performed with glass microneedles controlled by a Narishige micromanipulator (MO-202). Before they were used, microneedles and microcentrifuge tubes were exposed to ultra violet light for 30 min. Five copies of the structurally aberrant chromosomes of case 1 and 3 and the two chromosomes involved in the *de novo* translocation of case 2 were dissected. The chromosomes were transferred to a 20 µl collection drop (containing 250 µg/ml proteinase-K) in a 0.5 ml thin walled microcentrifuge tube. A fresh microneedle was used for each chromosome. Before DOP-PCR the collection drop was incubated at 37 °C for 30 min (proteinase-K treatment) and at 90 °C for 10 min (proteinase-K inactivation). The universal primer 5'CCGACTCGAGNNNNNNATGTGG 3' was used in the DOP-PCR as suggested by Telenius et al.¹⁵. The PCR buffer contained 100 µM of each dNTP, 2.5mM MgCl₂, 50mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatine, 0.1% (v/v) Nonidet P-40, 0.1% (v/v) Tween-20, 1.5 µM universal primer and 0.5 U super Taq DNA polymerase (HT Biotechnology) in a final volume of 50 µl. For initial denaturation the mixture was heated to 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 at 94 °C, 1 min at 56 °C, 3 min at 72 °C with a final extension step at 72 °C for 10 min. The DOP-PCR experiments contained a negative control consisting of all PCR components except microdissected DNA and a positive control with 25 pg total human DNA. PCR reactions were performed in a Biometra personal cycler with a heated lid. PCR products were stained with ethidium bromide and analysed for yield and probe size (200-900 base pairs) on an agarose gel. Experiments were proceeded only if DNA synthesis was not apparent in the negative control. The PCR product was precipitated and purified with 4 M ammonium acetate and isopropanol, washed with ethanol (70%) and dissolved in 50 µl T0,1E buffer (10 mM TRIS-HCl/0,1 mM Na₂EDTA, pH 8.0).

Fluorescence in situ hybridization (FISH).

PCR products were labeled by nick-translation with biotin-14-dATP (BioNick labeling system (Gibco BRL)). Metaphase spreads for FISH were prepared from PHA-stimulated lymphocytes using standard methanol: acetic acid (3:1) fixation. *In situ* hybridization was performed following the protocol by Guan et al.¹⁶ with only minor modifications. Briefly, for hybridization, 200 ng probe was used in 10 µl hybridization mixture containing 50% formamide, 2xSSC, 10% dextran sulphate, 50mM phosphate, 20 µg human Cot 1 DNA (Gibco BRL). Probe and Cot DNA were denaturated at 70 °C for 5 min and reannealed at 37 °C for 90 min. Slides were denaturated in 70% formamide, 2xSSC (pH 7.0) at 70 °C for 3 min, dehydrated, and hybridized overnight in a moist chamber at 37 °C with the probe. Slides were then washed four times in 2xSSC (pH7.0) at 45 °C for 5 min, followed by one wash in PN buffer [0.1 M sodium phosphate, 0.1% (v/v) Nonidet P-40, pH 8.0] at 45 °C for 10 min. The hybridization signal was detected by three layers of fluorescein-avidin DCS (Vector) amplified by two layers of biotinylated anti-avidin D (Vector), followed by two washes with 4xSSC, 0.5% Tween and one wash with PBS. Slides were dehydrated and counterstained with DAPI/PI in antifade solution. Analysis was carried out using a Zeiss Axiophot

microscope and photographs taken with Scotch chrome 640 ASA colour slide film.

Results

In case 1, analysis of 25 G-banded high-resolution metaphases disclosed a deletion of either region 3q23-3q25 or 3q25-3q26.2 (fig. 1A). After microdissection and reverse chromosome painting with the DOP-PCR probe generated from the aberrant chromosome 3, the deleted chromosome was completely painted in metaphases of the patient but the normal chromosome 3 showed no hybridization signal in region 3q23-3q25 (Fig. 2A). The parents had normal karyotypes.

In case 2 examination of 17 G-banded high-resolution metaphases revealed a translocation between the long arms of chromosomes 14 and 18 along with a deletion of either band 14q23 or band 18q12.3 (fig. 1C and 1D). FISH with paints specific for chromosome 14 and 18 indicated that the missing chromosome band was not translocated to any other chromosome (not shown). After hybridization of the probe generated by DOP-PCR of the microdissected derivative chromosomes 14 and 18 to metaphases of the patient, the der(14), der(18) and the normal chromosome 14 were completely painted. However, the normal chromosome 18 did not display a signal in band 18q12.3 (fig. 2B). The parents had normal karyotypes.

In case 3 examination of 25 high-resolution G-banded metaphases revealed a Robertsonian translocation between the chromosomes 14 and 15 and a deletion of region 15q11-q13 in the chromosome 15 not involved in the translocation (fig. 1B). Analysis of the father's chromosomes showed that he was a carrier of the translocation, whereas the mother had a normal 46,XX karyotype. Following reverse chromosome painting with the generated DOP-PCR probe, the deleted chromosome 15 was completely painted and band 15q12 in the chromosome 15 involved in the Robertsonian translocation (fig. 2C) displayed no fluorescent signal.

TABLE 1: CALCULATION OF THE AMOUNT OF MISSING BASE PAIRS IN THE ABERRANT CHROMOSOMES

	Rel. length ¹	Base pairs	Deletion ²	Base pairs
Chromosome 3	7.06	212 x 10 ⁶		
Case 1			10.25 %	22 x 10 ⁶
Chromosome 15	3.36	100 x 10 ⁶		
Case 3			6.0 %	6 x 10 ⁶
Chromosome 18	2.76	83 x 10 ⁶		
Case 2			10.8 %	9 x 10 ⁶

¹: Relative length in percentage of the total haploid autosome length (ISCN, 1985)²⁶.

²: Calculated deleted region expressed as a percentage of the entire length of the chromosome involved.

Discussion

In this study we demonstrate that a deleted region in an aberrant chromosome can be detected by microdissection of the aberrant chromosome with subsequent DOP-PCR and *in situ* hybridization of the generated probe to metaphase cells derived from the patient. The deleted region is immediately recognizable in the microscope owing to the lack of a fluorescent signal in the normal chromosome.

Microdissection of G-banded human metaphase chromosomes has been used by several laboratories to generate region-specific libraries. Restriction endonuclease digestion and DNA ligation steps were used by Johnson et al.¹⁷, Lüdecke et al.¹⁸, Davis et al.¹⁹ and Lengauer et al.²⁰ in their studies. Meltzer et al.³ and Deng et al.⁴ developed a technique that eliminated the microchemical procedure using the PCR product for cloning. Subsequently, Guan et al.²¹⁻²² used DOP-PCR probes generated from microdissected chromosomal material for chromosome (region) painting. Micro-FISH is employed today in clinical cytogenetics laboratories to characterize unidentifiable chromosome abnormalities, such as marker chromosomes and complex chromosomal rearrangements.

In this study micro-FISH was applied to demonstrate the loss of chromosomal material in aberrant chromosomes and to delineate the deleted region more precisely. The complete painting of the derivative chromosomes in the experiments provides evidence that the chromosomes indeed had been microdissected and multiplied by DOP-PCR completely. In case 1, cytogenetic analysis revealed a deletion in the long arm of one of the chromosomes 3; however, it could not be determined whether the chromosome region involved was 3q23-q25 or 3q25-q26.2. Micro-FISH allowed us to identify the deleted region as 3q23-q25 based on the lack of a fluorescent signal in this region in the normal chromosome 3. Therefore, in combination with the G-banding results, the consensus karyotype for case 1 would be: 46,XX,del(3)(q23q25). This is in accordance with the clinical picture presented by the patient since blepharophimosis in combination with ptosis and epicanthus inversus is frequently reported in patients with a deletion or break in 3q23 (Jewett et al.²⁴; Small et al.²⁵).

Case 2 is a patient with a *de novo* translocation involving chromosomes 14 and 18 along with a deletion of either chromosome band 14q23 or 18q12.3. Hybridization of the DOP-PCR probe to metaphase chromosome preparations derived from the patient showed a lack of fluorescence in the normal chromosome at band 18q12.3 indicating that he has an unbalanced 46,XY,t(14;18)(14pter->14q22::18q21.1->18qter;18pter->18q12.2::14q23->14qter) karyotype in which band 18q12.3 is deleted.

Case 3 is a girl with a Robertsonian translocation, der(14;15)(q10;q10) and a deletion of region 15q11-q13 in the second chromosome 15. Owing to the involvement of the normal chromosome 15 in the translocation, the deleted chromosome 15 could be traced without confusion. This patient, with a microdeletion of band 15q12 that is frequently reported in patients with Prader-Willi or Angelman syndrome, was included in this study to test whether this new application of micro-FISH is powerful enough to detect microdeletions.

The haploid human genome comprises 3×10^9 base pairs and 3.36% of them are present in one chromosome 15 (ISCN, 1985)²⁶. Chromosome band 15q12 constitutes

Figure 2: **A:** Metaphase cell of case 1 after FISH with the DOP-PCR probe generated from the aberrant chromosome 3. **B:** Metaphase cell of case 2 after FISH with the DOP-PCR probe generated from both chromosomes involved in the 14q;18q translocation. **C:** Metaphase cell of case 3 after FISH with the DOP-PCR probe generated from the aberrant chromosome 15. Arrows indicate the "deleted regions".

about 6% of chromosome 15; consequently, the deletion comprises about 6×10^6 base pairs (table 1). After reverse chromosome painting with the DOP-PCR probe, the deletion was clearly recognizable owing to the lack of fluorescent signal in the corresponding region of the translocation chromosome. Indeed, when chromosome quality is poor, as is true of many hematological and solid tumors, deletions can be characterized more precisely through hybridization of the generated DOP-PCR probe to high-resolution chromosomes of a control individual.

This paper has shown the power of micro-FISH as a tool for the characterization of chromosome abnormalities. The primary advantages of this technique lie in its simplicity and speed. The whole process of microdissection, DOP-PCR, probe labeling and reverse painting can be completed within three days. Furthermore, although probes of virtually all chromosome bands exist and deletion affirmation is possible with band-specific cosmids, micro-FISH has the advantage that laboratories do not have to possess a gigantic stock of probes. The colour images for this report were made with a Zeiss Axiophot microscope and illustrate that this technique produces perfectly adequate results without image enhancement.

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**CHARACTERIZATION OF A DE NOVO UNBALANCED TRANSLOCATION
t(14q18q) USING MICRO-FISH.**

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We report on a patient with a de novo translocation between the long arm of chromosomes 14 and 18. The translocation was studied using microdissection in combination with fluorescence in situ hybridization (micro-FISH). Five copies of the chromosomes involved in the translocation were isolated by microdissection and amplified by means of degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR). Reverse chromosome painting with the biotin-labeled PCR product showed that part of the q-arm of chromosome 18 had no signal. The deletion was characterized further by FISH with band-specific probes and it was concluded that the rearrangement was unbalanced: 46,XY,t(14;18)(14pter->14q22::18q21.1->18qter;18pter->18q12.2::14q22->14qter). The patient, who presented with psychomotor retardation, mild obesity, pes equinovarus, strabismus and facial anomalies, is compared with previously reported patients with an interstitial deletion of band 18q12.

Introduction

Microdissection in combination with fluorescence *in situ* hybridization (micro-FISH) involves the physical dissection of GTG-banded chromosomes followed by degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) and subsequent FISH with the probe obtained. This method, developed by Meltzer et al.¹ has been successfully applied in clinical cytogenetics to characterize unidentifiable chromosome abnormalities (Ohta et al.²), chromosome rearrangements (Zhang et al.³; Engelen et al.⁴), and marker chromosomes (Thangavelu et al.⁵; Viersbach et al.⁶; Sun et al.⁷; Muller-Navia et al.⁸; Engelen et al.⁹). Loss of chromosomal material was demonstrated with micro-FISH by Rubtsov et al.¹⁰, Guan et al.¹¹ and Engelen et al.¹².

In this study micro-FISH was used to characterize a *de novo* translocation between chromosomes 14 and 18, that was found in an obese, 3-year-old boy with psychomotor retardation and other anomalies. From high-resolution banding studies it was clear that the translocation was unbalanced. Although chromosome painting (Lichter et al.¹³; Pinkel et al.¹⁴) allows the rapid identification of translocations, this technique was insufficient to determine precisely which chromosome band was deleted. However, the application of microdissection and reverse chromosome painting (Carter et al.¹⁵) resulted in the successful identification of the deleted region in this unbalanced *de novo* translocation. FISH with band-specific probes was applied to characterize the deleted region and the breakpoints more precisely.

Material and methods

Clinical report

The patient, a 3-year old boy, was the third child of non-consanguineous, healthy parents. During pregnancy fetal movements were reduced. He was born at term by vacuum extraction, with severe postnatal acidosis (arterial pH 6.80). Birth weight was 2690 g, and length 48 cm. He had a club foot at the right side, for which he was successfully treated. Psychomotor development was retarded, with generalized hypotonia and lack of motoric development until the age of 10 months. He sat without support at the age of 13 months and stood at 21 months. When examined at 2 years and 3 months, we saw a friendly child with a length of 95 cm (90th centile), weight of 15 kg (75th centile) and a head circumference of 50.5 cm (50th-75th centile). He had plagiocephaly, a narrow forehead, deep set eyes, epicanthal folds, convergent strabismus, small mouth with a thin upper lip, high narrow palate, large and broad hands and small and puffy feet. He also had wide set nipples. In the left lumbal region a hairy patch of 4 cm in diameter was seen. There was bilateral cryptorchidism but otherwise his genitalia were normal. There was mild axial hypotonia and he could not walk without support. He spoke only four words. There were no behaviour problems. A magnetic resonance image (MRI) of the brain was normal, MRI of the spinal cord showed a stenosis at level L4-L5. The findings of the most recent examination at 3 years of age are summarized in table 1 and his distinct craniofacial appearance is illustrated in figure 1.

Pict.1: Picture of the patient at the age of three years

Cytogenetics, microdissection and FISH

Chromosomes were prepared from peripheral blood lymphocytes using the synchronization method of Dutrillaux and Viegas-Pequignot¹⁶ with minor modifications. High-resolution banding was performed by treatment with trypsin followed by staining with Giemsa to obtain a GTG-banded pattern.

Fig. 1 : Partial karyotype presenting normal GTG-banded chromosomes 14 and 18 and the chromosomes involved in the translocation. Arrows point to the breakpoints, asterisks mark the points of reunion.

For microdissection, routinely fixed cell suspensions stored at -20°C were used to prepare metaphase spreads on coverslips. Dissection of the translocation chromosomes was performed with glass microneedles controlled by a Narishige micromanipulator (MO-202). Five copies of both the derivative chromosome 14 and 18 were collected in a $20\text{ }\mu\text{l}$ collection drop; amplification of the dissected DNA by DOP-PCR was performed as described previously (Engelen et al.¹²). The PCR product was precipitated, dissolved in $50\text{ }\mu\text{l}$ T0,1E buffer (10 mM TRIS-HCl/ $0,1\text{ mM}$ Na2EDTA, pH 8.0) and labelled with biotin-14-dATP by nick-translation. FISH with the DOP-PCR product was performed following the protocol of Guan et al.¹⁷ with minor modifications.

In this study the following band specific probes were used:

Probe	Localization	References
SCW 0201C	18q12	Nakashima et al. ¹⁸
SCW 0307E	18q12	Nakashima et al. ¹⁸
D18S46	18q21.1	Boghossian-Sell et al. ¹⁹
D18S41	18q21.2-18q21.3	Boghossian-Sell et al. ¹⁹

Probes were labelled with biotin-14-dATP by nick-translation and for FISH with these probes the protocol of Lichter et al.²⁰ was followed. Analysis was carried out using a Zeiss epi-fluorescent Axiophot microscope and photographs taken with Scotch chrome 640 ASA colour slide film. Chromosome painting was performed according to the manufacturer's specifications.

Results

Analysis of GTG-banded metaphases demonstrated a translocation between chromosomes 14 and 18. From banding patterns it was concluded that either band 14q23 or band 18q12.3 was deleted (fig. 2). Chromosome 14 and 18 specific paints were used to show that the missing chromosome band was not translocated to another chromosome (fig. 3A and 3B). After hybridization of the DOP-PCR probe to normal metaphase chromosomes both chromosomes 14 were found to be completely painted. However, chromosomes 18 lacked fluorescence in region 18q12 (fig. 3C). After hybridization of the DOP-PCR probe to metaphases of the patient, the der(14) and der(18) were completely painted giving evidence that these chromosomes had been completely microdissected and multiplied by DOP-PCR. Also the normal chromosome 14 was completely painted. However, as expected, chromosome 18 showed no signal in band 18q12 (fig 3D). FISH with probes SCW 0201C (fig. 4A) and SCW 0307E (not shown) to metaphases of the patient confirmed these results, as only the normal chromosome 18 showed a fluorescence signal. After FISH with probes D18S46 (fig. 4B) and D18S41 (not shown) to metaphases of the patient of both probes two fluorescence signals were visible; one signal on chromosome 18 and one signal on the der(14). This indicates that the distal breakpoint in the q-arm of chromosome 18 is located proximally to D18S46 and D18S41. The FISH results combined with the GTG-banding show that the patient has an unbalanced 46,XY,t(14;18)(14pter->14q22::18q21.1->18qter;18pter->18q12.2::14q23->14qter) karyotype. Chromosomes of the parents were normal.

Fig. 3A and 3B: Metaphase chromosomes of the patient after FISH with a chromosome 14 (A) and chromosome 18 (B) specific paint. **Fig. 3C:** Metaphase of a normal male after FISH with the DOP-PCR probe generated from the chromosomes involved in the translocation. The arrows are pointing to the "deletion" in chromosome 18. **Fig. 3D:** Metaphase of the patient after FISH with the DOP-PCR probe generated from the chromosomes involved in the translocation. Small arrow marks the "deletion" in chromosome 18.

Fig. 4A: Metaphase chromosomes of the patient after FISH with probe SCW 0201C. **Fig. 4B:** Metaphase chromosomes of the patient after FISH with probe D18S46.

Discussion

In this study micro-FISH was successfully applied to demonstrate loss of chromosomal material in a patient with a *de novo* translocation involving chromosomes 14 and 18. FISH with band-specific probes confirmed the loss of chromosomal material and characterized the breakpoints more precisely. To the best of our knowledge, this case represents the first report of del(18q12.3) due to a *de novo* translocation.

In Table I, the clinical findings of our case are compared with 13 previously reported cases with deletion of 18q12. The first 4 patients have a deletion of 18q12 due to unbalanced transmission of a familial insertion translocation (11;18)(p15;q11q21), the remaining 9 cases have *de novo* deletions. In all patients with this deletion moderate to severe mental retardation is reported. In addition, ataxia, seizures or EEG abnormalities, hypotonia and a tendency to mild obesity are commonly reported. Craniofacially, the younger patients reported in the literature bear a striking resemblance to our patient. They share their mild facial anomalies that include prominent forehead with short nose, epicanthal folds, deep-set eyes, abnormal mouth with thin upper lip, and ear anomalies. Table I shows that the clinical findings of patients with a larger deletion and breakpoints in 18q11 and 18q21 do not differ significantly from the clinical findings of patients with deletion of only chromosome band 18q12.3. The smallest region of overlap for these 14 cases is chromosome band 8q12.3 (figure 5). As could be expected, this clinical picture does not resemble the terminal deletion 18(q21->qter) phenotype which results in the de Grouchy syndrome (de Grouchy and Turleau²¹).

TABLE 1. Clinical findings in 14 cases with deletions involving chromosome band 18q12.3.

References:	1	1	1	1	2	3	4	5	5	6	7	7	7	our
	a	b	c	d			a	b		a	b	c		pt.
Proximal breakpoint	q11	q11	or q12.2	q12.1			q12.1		q12.2		q12.2		q12.2	
distal breakpoint	q21	q12.2	or q21.	q21.1			q12.3		q21.1		q21.1		q21.1	
Age of patient	8mo	42	40	38	1	3	2	3	6	7	4	2	4	3
Mental retardation	+	+	+	+	+	+	+	+	+	+	+	+	+	
Hypotonia	-	o	o	+	+	+	-	+	+	+	+	+	-	+
High forehead	+	-	+	+	+	+	+	-	+	+	-	-	+	-
Epicanthal folds	o	o	o	o	+	o	+	+	+	-	-	+	+	+
Deep set eyes	+	+	+	+	o	+	-	+	+	+	o	o	o	+
Flat nasal bridge	+	-	-	-	+	+	+	+	+	+	o	o	+	+
Short nose	+	-	-	-	+	+	-	o	o	+	+	+	+	+
Small midface	+	+	+	+	o	+	-	+	+	o	+	+	+	-
Wide philtrum	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Abnormal mouth	+	+	o	o	+	+	-	+	+	+	+	o	o	+
Abnormal ears	+	+	+	+	+	+	-	+	+	o	+	+	+	+
Strabismus	o	o	o	o	+	o	-	+	-	-	-	+	+	+
Abnormal EEG /														
Seizures	-	-	+	-	+	+	-	+	+	o	+	+	+	+
Behaviour problems	+	+	+	+	+	-	-	+	+	+	+	-	+	-

+: present, -: not present, o: not documented.

References:

- 1: Chudley et al.²⁵, patients III/10 (a), II/4 (b), II/5 (c), II/6 (d).
- 2: Wilson et al.²⁶.
- 3: Krasikov et al.²⁷.
- 4: Suhr et al.²⁸.
- 5: Poissonnier et al.²⁹ patients 1 (a) and 2 (b).
- 6: Wilson and Al Saadi³⁰.
- 7: Schinzel et al.³¹ patients 1 (a), 2 (b) and 3 (c).

The most likely mechanism for the production of the reported abnormality involves 3 chromosomal breaks: 2 in chromosome 18, producing a fragment consisting of 18q12.3, and a simultaneous break in 14q22. Although the clinical picture of our patient is most likely caused by deletion of chromosome band 18q12.3, involvement of a small submicroscopic deletion or a disrupted gene (sequence) at the breakpoint in chromosome 14 cannot be completely ruled out. This is supported by data of Kousseff et al.²², Fryns et al.²³ and Warburton²⁴ who report that mental retardation is more frequently present in patients with a *de novo* translocation than in carriers of familial translocations.

Fig. 5 : Ideogram of chromosome 18 and schematic representation of the deleted region in the patients from table I. SRO: smallest region of overlap.

In summary, it seems that patients with interstitial deletion 18q12 lack major malformations and have a normal lifespan. Consistent clinical findings in young patients are mental retardation, hypotonia, epicanthal folds, short nose with flat bridge, wide philtrum, abnormal mouth and ears, deep-set eyes, mild obesity and abnormal EEG. Furthermore, this study illustrates the value of micro-FISH for relatively rapid and precise characterization of an aberrant chromosome. That this is possible in three days makes it a perfectly suitable method for the clarification of chromosome abnormalities in areas such as prenatal diagnosis.

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DUPLICATION OF CHROMOSOME REGION 15Q11-15Q13 IN A PATIENT WITH SIMILARITIES TO PRADER-WILLI SYNDROME CONFIRMED BY REGION-SPECIFIC AND BAND-SPECIFIC FISH.

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We report on a patient presenting with mental retardation and obesity and a proximal duplication of chromosome 15. The patient shared some clinical signs with Prader-Willi syndrome. With a region specific paint, generated by microdissection, a duplication in region 15q11.2-q13 was shown to be present. Subsequently, FISH with probes localized to chromosome region 15q11.2-q12 further characterized this chromosome aberration and a duplication of sequences which are frequently deleted in Prader-Willi and Angelman syndrome was demonstrated.

Introduction

Prader-Willi syndrome (PWS) and Angelman syndrome (AS) are mental retardation disorders which are caused by the loss of function of genes proximal on chromosome 15 (q11-q13). The paternal origin of deletions in PWS, as well as the occurrence of maternal uniparental disomie (UPD), suggest that a gene(s) associated with PWS is active on the paternal chromosome only. In AS, maternal deletions and paternal UPD suggest that the AS gene(s) is active on the maternal chromosome only. This different parental origins of deletions and UPD strongly implicate genomic imprinting for this chromosome region (Nicholls¹).

Less frequently, proximal duplication in the long arm of chromosome 15 has been described in patients with Prader-Willi or "Prader-Willi-like" syndrome (Fuhrmann-Rieger², de France et al.³, Berry et al.⁴, Pettigrew et al.⁵, Rauch and Nevin⁶, Li et al.⁷) in a patient with similarities to Angelman syndrome (Clayton-Smith et al.⁸) and in mentally retarded patients without the typical clinical features of these syndromes (Pettigrew et al.⁵, Brookwell and Veleba⁹, Turolla et al.¹⁰, Shohat et al.¹¹, Ludowese et al.¹², Hirsch et al.¹³, Crawford et al.¹⁴). Beside that, a proximal duplication is known which in phenotypically normal individuals is segregating as a polymorphism (Li et al.⁷, Brookwell and Veleba⁹, Turolla et al.¹⁰, Ludowese et al.¹², Hirsch et al.¹³, Jalal et al.¹⁵, Jalal et al.¹⁶). although transmission from a phenotypically normal parent to an abnormal child has also been reported (Berry et al.⁴, Rauch and Nevin⁶, Li et al.⁷, Brookwell and Veleba⁹, Turolla et al.¹⁰, Shohat et al.¹¹, Ludowese et al.¹²).

This report is on the delineation of a proximal 15q duplication in a 12-year-old mentally retarded boy with some of the typical clinical features of PWS. Cytogenetic analysis of G-banded chromosomes disclosed a de novo duplication in the proximal region of chromosome 15. With a region 15q11-q13 specific paint, that was made by microdissection and subsequent degenerate oligonucleotide primed-PCR (DOP-PCR), metaphases of the patient were painted. Furthermore, FISH with locus-specific probes was applied to characterize the duplication more precisely.

Material and methods

Clinical report

The patient is the second child of healthy parents. His elder sister is normal. Pregnancy and birth were unremarkable. No early hypotonia, nor feeding problems existed. Development was generally delayed and special schools were necessary. Weight was at the high percentiles from the first year of life. At the age of 12 years, the proband was referred because of mild mental retardation and obesity, suggesting PWS. Clinical examination showed an obese boy with some aspects of the PWS phenotype (fig. 1). Height was between 10 and 25th percentile, weight for height was well above the 97th percentile. Head circumference was normal. Minor dysmorphism of the face included high forehead with small biparietal diameter, slightly upslanting palpebral fissures and myopia. A tendency for overconsumption was present. Saliva was thick and the teeth were very bad looking because of caries. Hands were small (<P3) and soft. Genital

development was early pubertal, penis length being 5 cm. Skin picking was apparent and according to the parents tantrums were common. Apart from the early data, clinical and behavioural phenotype of the proband were judged to be compatible with the diagnosis of PWS.

Major criteria (1 point each)	Present patient
Infantile central hypotonia	-
Infantile feeding problems/failure to thrive	-
Rapid weight gain between 1 and 6 years	+
Characteristic facial features	+
Hypogonadism: genital hypoplasia, pubertal deficiency	-
Developmental delay/mental retardation	+
Minor criteria (1/2 point each)	
Decreased fetal movement and infantile lethargy	-
Typical behavioural problems	-
Sleep disturbance/sleep apnoea	-
Short stature for the family by age 15 years	-
Hypopigmentation	+
Small hands and feet for height age	+
Narrow hands with straight ulnar border	+
Esotropia, myopia	+
Thick, viscous saliva	+
Speech articulation defects	-
Skin picking	+

Table 2: Summary of the clinical diagnostic criteria for Prader-Willi syndrome (adapted from Holm et al.¹⁷), and the clinical signs present in our patient. The diagnosis should be strongly suspected in children under 3 years of age with five points, three from major criteria, or in those above 3 years with eight points, four from major criteria.

Fig. 1:
The proband, a 12-year-old boy.

Cytogenetics

Blood lymphocyte cultures for chromosome studies were synchronized overnight with thymidine, treated with 5-BrdU for 6 hours and ethidiumbromide for 1.5 hours during the last S-phase before harvest (Dutrillaux et al.¹⁸). High resolution banding was performed by treatment with trypsin followed by staining with Giemsa to obtain a GTG-banded pattern.

Microdissection and fluorescence in situ hybridization

A region 15q11-q13 specific paint was produced by microdissection of an isochromosome 15 (15pter->q13::q13->15pter). Five copies of this marker chromosome were dissected and amplified by DOP-PCR as described previously (Engelen et al.¹⁹). In brief, the dissected chromosomal material was transferred to a 20 µl collection drop (containing 250 µg/ml proteinase-K) in a 0.5 ml thin walled microcentrifuge tube. Next, the collection drop was incubated at 37 °C for 30 min (proteinase-K treatment) and at 90 °C for 10 min (proteinase-K inactivation). The universal primer 5'CCGACTCGAGNNNNNNATGTGG 3' was used in the DOP-PCR as suggested by Telenius et al.²⁰ The PCR buffer contained 100 µM of each dNTP, 2.5mM MgCl₂, 50mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatine, 0.1% NP-40, 0.1% Tween-20, 1.5 µM universal primer and 0.5 U super Taq DNA polymerase (HT Biotechnology) in a final volume of 50 µl. For initial denaturation the mixture was heated to 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 at 94 °C, 1 min at 56 °C, 3 min at 72 °C with a final extension step at 72 °C for 10 min. The DOP-PCR experiments contained a negative control and experiments were proceeded only if DNA synthesis was not apparent in this control. PCR product was precipitated and purified with 4 M ammonium acetate and isopropanol, washed with ethanol (98%), dissolved in 50 µl T0,1E buffer (10 mM TRIS-HCl/0,1 mM Na₂EDTA, pH 8.0) and biotin labeled by nick translation. FISH with this region specific paint was performed following the protocol of Guan et al.²¹ with only minor modifications. Additionally, the following locus specific probes were used:

Locus	Probename	Size (kb)	Chromosome-region	Reference
D15S11	A156E1	250	15q11.2-q12	Kuwano et al. ²²
GABRB3	B25E9	150	15q11.2-q12	Kuwano et al. ²²

The probes were biotin-14-dUTP labeled by nick translation and for FISH the protocol of Lichter et al.²³ was followed. The slides were examined with a Zeiss Axiophot fluorescence microscope and photographed using Scotchchrome 640 ASA colour slide film.

Methylation analysis

Genomic DNA was digested with Hind III and Hpa II. Fragments were separated by agarose gel electrophoresis and analysed by Southern blot hybridization with probe PW71B for D15S63 (Dittrich et al.²⁴).

Fig.2A: Partial karyotype showing G-banded chromosome 15 (left), chromosome 15 with duplication q11.2-q13.1 (right) and their idiograms. **Fig.2B:** FISH with the region 15q11-15q13 specific paint to a metaphase of the patient. Small and large arrows point to normal chromosome 15 and chromosome 15 with proximal duplication. **Fig.2C and 2D:** FISH with probe A156E1 and B25E9 to a metaphase of the patient. Small and large arrows point to normal chromosome 15 and chromosome 15 with proximal duplication.

Fig. 3: Partial karyotype showing G-banded chromosomes 15 of the mother (left), patient (middle) and father (right). Arrows point to chromosome 15 with the duplication.

Results

Cytogenetic analysis of G-banded chromosomes revealed a proximal duplication in one chromosome 15 in all metaphases (fig. 2A). FISH with the region-specific paint to metaphases of the patient showed that the fluorescence signal in the aberrant chromosome 15 was about two times the size of the signal in the normal chromosome 15 (fig. 2B). FISH with locus specific probes A156E1 and B25E9 showed, for both probes, two pairs of fluorescent spots on the aberrant chromosome 15 and one pair of spots on the normal chromosome 15 (fig. 2C and 2D respectively). The parents of the patient had normal karyotypes. Comparison of the polymorphic regions of chromosomes 15 of the parents and the patient revealed that the aberrant chromosome was inherited from the father (fig. 3). Methylation status analysis showed that a maternal 6.6 kb band as well as a paternal 4.7 kb band were present, demonstrating normal contribution of parental chromosomes 15.

Table 1: Clinical features of patients with proximal duplication 15q and results of molecular studies.

Author	Age	Clinical features and results of molecular studies.
Li et al. ⁶	0	Prader-Willi syndrome. GABRB3 duplicated.
Li et al. ⁶	?	Phenotypically normal mother of previous patient. GABRB3 duplicated.
Li et al. ⁶	?	Phenotypically normal father and grandfather of previous patients. GABRB3 duplicated.
Clayton-Smith et al. ⁷	6y	Ataxia, mild mental retardation and speech delay. D15S10 duplicated.
Hirsch et al. ¹²	4y	Mental retardation, absence of speech and autistic disorder. D15S11-SNRPN-GABRB3 duplicated.
Hirsch et al. ¹²	?	Intellectually normal mother of previous patient. D15S11-SNRPN-GABRB3 duplicated.
Crawford et al. ¹³	8y	Moderate mental retardation and autistic behaviour. D15S10-D15S11-SNRPN-GABRB3 duplicated
Jalal et al. ¹⁴	0	Phenotypically normal neonate ascertained by amniocentesis. D15S11-GABRB3 single copies.
Jalal et al. ¹⁴	?	Phenotypically normal father of previous patient D15S11-GABRB3 single copies.
Present patient	12y	Obesity, mental retardation, small soft hands, myopia, caries, tantrums, skin picking. D15S11-GABRB3 duplicated.

Discussion

PWS is generally caused by the absence of the paternal chromosome 15q12 region due to (micro)deletion, uniparental disomy or abnormal DNA methylation due to a mutation in the imprinting centre (Buiting et al.²⁵). Occasionally the critical region involved is affected by other chromosomal rearrangements like translocations, inversions or duplications.

In our patient with similarities to PWS, high resolution banding showed a proximal duplication of chromosome 15. As whole chromosome painting would not differentiate between a duplication of 15q11.2-q13 and a proximal insertion of another region of chromosome 15, we decided to paint the patients' metaphases with a region-specific paint. With this paint, the aberrant chromosome 15 showed a visually increased fluorescent signal in the proximal region, proving that region 15q11-q13 was involved in the duplication.

Kuwano et al.²² used probes A156E1 and B25E9, specific for two loci in region 15q11.2-q12, to study 8 patients with PWS and 3 patients with AS. In all eleven patients a deletion for the region containing these probes was demonstrated. FISH with these probes to metaphases of our patient confirmed the duplication, as the aberrant chromosome 15 showed two pairs of fluorescent spots in the proximal region. Methylation analysis of locus D15S63 with probe PW71 however detected a normal biparental methylation pattern. In patients with proximal duplication in chromosome 15 reported in the literature, only the past four years molecular genetic studies or FISH with locus specific probes have been performed. Clinical features of these patients and the results of the molecular studies are listed in table 1. Clayton-Smith et al.⁸ were the first to demonstrate a duplication of D15S10 in a patient with similarities to AS. Later, FISH with specific probes for D15S11, D15S10, SNRPN and/or GABRB3 (fig. 4) was used to prove a duplication of (part of) 15q11-q13 in aberrant chromosomes 15 of a child with PWS (Li et al.⁷), a child with autism (Hirsch et al.¹³) and a child with moderate mental retardation and autistic behaviour (Crawford et al.¹⁴).

Fig. 4: Genetic map of chromosome region 15q11-q13, (modified from ref. 32) illustrating the position of genetic markers, probes A156E1, PW71 and B25E9, critical regions involved in PWS and AS and imprinting centre (IC).

Finally, Jalal et al.^{15,16} used FISH to determine the content of duplicated proximal regions of chromosome 15 in normal persons and showed that single copies of D15S11 and GABRB3 were present. As the phenotype of patients with a chromosome 15q11-q13 duplication is rather variable it might be reasonable to assume that, like in PWS and AS, the parental origin of the duplication chromosome influences the phenotype of the patients. The patient described by Clayton-Smith et al.⁸ has a maternally derived chromosome 15 duplication and similarities to AS, our patient has a paternally derived duplication and similarities to PWS. Several imprinted genes have been identified in chromosome region 15q11-q13: SNRPN (Glenn et al.²⁶), IPW (Wevrick et al.²⁷), ZFP127 (Driscoll et al.²⁸), and two anonymous transcripts (PAR-1 and PAR-5) (Sutcliffe et al.²⁹). Altered dosage of (one of these) genes involved in PWS or modified gene products might be responsible for the specific subset of clinical features in our patient. This is also in agreement with previous observations in patients with triplication of 15q11-q13 studied with molecular techniques (Holowinsky et al.³⁰, Schinzel et al.³¹). In these patients the increased dosage of genes in the PWS/AS critical region caused an abnormal phenotype, depending on the copy number and the parental origin. In conclusion, a proximal duplication in chromosome region 15q11-q13 was demonstrated with a region-specific paint and characterized further with locus-specific probes. In our opinion this duplication of sequences, frequently deleted in PWS and AS, causes the Prader-Willi like phenotype of the patient. Molecular analysis of the DNA methylation pattern disclosed no abnormality in this patient, indicating that chromosome analysis should always be applied in patients with clinical findings suggestive for PWS or AS. Furthermore, molecular analysis or FISH should be applied on patients with a proximal duplication in chromosome 15 to distinguish between a duplication of D15S10, D15S11, SNRPN or GABRB3 or a duplication or amplification of part of a region proximal of 15q12 which seems a heritable and normal G-band polymorphism (Jalal et al.^{15,16}).

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GENERAL DISCUSSION AND CONCLUSIONS

Microdissection, the technique to pick up chromosomal regions with a glass needle, was introduced some 15 years ago by Scalenge et al.¹. It was used for the cloning in bacteria of genomic sequences from *Drosophila* salivary gland polytene chromosome bands. The introduction of the polymerase chain reaction (PCR) and sequence independent DNA amplification enabled Lüdecke et al.² to amplify dissected chromosomal material *in vitro*. Technical improvements allowed to reduce the number of dissected fragments from >100 (Bates et al.³) to approximately 25 and to use GTG-banded chromosomes for microdissection (Senger et al.⁴). Meltzer et al.⁵ amplified microdissected chromosomal material using a degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) and directly used the DOP-PCR product for reverse chromosome painting (micro-FISH).

In micro-FISH, (microdissection combined with fluorescence *in situ* hybridization) four equally important steps can be distinguished. (1) The physical dissection of GTG-banded chromosomes or chromosome parts using a dissection needle or a laser beam. The dissection is followed by (2) treatment of the dissected chromosomal material with proteinase-K, topoisomerase or heat to relax the supercoiled DNA of the metaphase chromosomes and (3) a degenerate oligonucleotide primed-polymerase chain reaction (DOP-PCR) to amplify the dissected DNA. Finally, (4) a reporter molecule (biotin or digoxigenin) is incorporated in the PCR product obtained and this PCR product is annealed to metaphase spreads from normal subjects to detect hybridization.

Micro-FISH is used in clinical cytogenetics to characterize chromosome aberrations like marker chromosomes, unbalanced chromosome translocations, complex chromosome rearrangements, deletions, duplications and inversions. As only five copies of a chromosome part have to be dissected to generate sufficient probe for an intense FISH signal, micro-FISH can also be used to analyse low grade mosaicisms. The whole process of microdissection, DOP-PCR, probe labeling and reverse painting can be completed within three days which makes this method perfectly suitable for prenatal diagnosis.

Marker chromosome characterization.

A marker chromosome (mar) is a structurally abnormal chromosome in which no part can be identified (ISCN 1995⁶). Mainly due to their small size and the lack of a distinct banding pattern marker chromosomes are often not identifiable by cytogenetic banding techniques. Constitutional markers are found with a frequency of 0.24/1000 in lifeborn individuals (Buckton et al.⁷) and 0.4 - 1.5/1000 at amniocentesis (Hook and Cross⁸, Sachs et al.⁹, Warburton et al.¹⁰). In mentally retarded patients the incidence of marker chromosomes is reported to be 3.27/1000 (Buckton et al.⁷). Over 80% of the markers originate from the pericentromeric regions of the acrocentric chromosomes (Maraschio et al.¹¹) and approximately 50% of these markers originate from the centromere region of chromosome 15 (Maraschio et al.¹¹, Blennow et al.¹²). Beside conventional staining techniques, FISH, often preceded by chromosome flow sorting and microcloning or DOP-PCR of the chromosomal material, has been used to identify marker chromosomes (Rauch et al.¹³, Plattner et al.¹⁴, Blennow et al.¹² and Crolla et al.¹⁵).

The most straightforward approach for identification of marker chromosomes is, without any doubt, micro-FISH. The advantages of micro-FISH to identify marker chromosomes above the methods mentioned before are that it is less time consuming than the microcloning procedures and in comparison to chromosome sorting, the size of the marker is not a limiting factor. Furthermore, only a small number of dissected chromosomes is needed and at least up to three years old fixed cell suspensions can be used making retrospective analysis possible. Finally, marker identification with centromere probes or paints discloses the chromosomal origin of the marker but not the chromosome region involved.

Analysis of complex chromosome rearrangements.

Complex chromosome rearrangements (CCRs) have been defined as structural rearrangements involving at least two chromosomes and three or more chromosome breakpoints (Pai et al.¹⁶). CCRs may be balanced or unbalanced, familial or de novo. Since interpretation of a CCR is often difficult when only standard GTG-banding techniques are used, FISH with whole chromosome paints, developed by Pinkel et al.¹⁷ and Lichter et al.¹⁸ is frequently used for characterization of a CCR. However, when a translocated region in a CCR is recognized due to its aberrant banding pattern, but complete characterization fails even with chromosome painting, then micro-FISH is the method of choice. Dissection of such a half-cryptic translocation followed by DOP-PCR and reverse painting will disclose the region(s) of origin of the dissected chromosome part as well as enable a better delineation of the breakpoints.

Deletion detection and breakpoint characterization.

Another group of chromosome aberrations in which micro-FISH can be used for characterization are deletions. Chromosomes in which a part is deleted are, in general, easy to recognize. However, it can sometimes be difficult to determine the breakpoints in deletion chromosomes exactly, when the interpretation is based only on the banding pattern. Microdissection of the deletion chromosome followed by DOP-PCR amplification and painting of the patient's chromosomes, will provide information about the position of the deleted region. The deleted region will not display a FISH signal in the patient's normal chromosome. However, the deleted chromosome must display a FISH signal over its complete length as a prove that it is dissected and amplified completely. From recent work of Rubstov et al.¹⁹ and Stevens-Kroef et al.²⁰ it became clear that deletions which look like terminal deletions with GTG-banding, are at least sometimes interstitial deletions. This finding will certainly lead to a re-interpretation of a number of terminal deletions.

Tumor cytogenetics

In many human cancers the presence of marker chromosomes or unbalanced translocations prevents complete karyotypic analysis. For the assessment of chromosome rearrangements micro-FISH is becoming increasingly important. Only a few copies of the aberrant chromosomes need to be dissected and are sufficient for reverse painting. Indeed, the need for only a few copies offers an advantage over previously described methods such as chromosome isolation by flow sorting (Telenius et al.²¹). Also in tumor cytogenetics, it is important to determine breakpoints precisely, because this creates the possibility to detect genes that play a role in tumor genesis. When chromosome quality is poor, as is true of many haematologic and solid tumors, deletions and breakpoints can be characterized more precisely through hybridization of the generated DOP-PCR probe to high resolution chromosomes of a control individual. Only very recently microdissection is used in conjunction with CGH (Liang et al.²²), which in tumor cytogenetics will enable to obtain tumor cell populations without contaminating stromal cells. Identification of regions with sequence gain or loss with micro-FISH and CGH provides opportunities to identify genes that may be involved in tumor development and disease progression.

Future perspectives

In chapter 4 it was demonstrated that it is possible to detect a microdeletion of approximately 6×10^6 base pairs when using high-resolution chromosomes for reverse painting. As the length of the chromosomes used for reverse painting is the limiting factor for the detection of submicroscopic deletions, it is logical to presume that when using longer chromosomes for reverse painting even submicroscopic deletions can be detected. The use of prematurely condensed chromosomes might be a logical choice to resolve this problem. This approach will certainly be valuable in the search for submicroscopic deletions which might exist in patients with a *de novo* translocation when they present with dysmorphisms.

When isolating DNA from a breakpoint for subsequent cloning, a substantial part of the isolated clones will not be useful as they are located at a considerable distance of the breakpoint. To overcome this problem, chromosome stretching (Claussen et al.²³) could be performed. With this technique it is possible to elongate chromosomes up to a resolution of 2000 bands or more per haploid autosome set. Consequently, after chromosome stretching followed by microdissection, it might be possible to construct a library of the dissected DNA that contains only clones of the breakpoint and close to the breakpoint.

Since the introduction of micro-FISH in 1992 a number of different methods have been developed to generate DOP-PCR probes (Guan et al.²⁴, Yokoyama et al.²⁵, Muller-Navia et al.²⁶, Engelen et al.²⁷). For the analysis of chromosome aberrations these methods seem to be equally efficient. However, until now it is unclear whether all the microdissected chromosomal material is amplified completely and thus present in the DOP-PCR product. Information about the saturation of the DOP-PCR product is

important when these probes are used for subsequent cloning. Reverse painting on DNA fibers (Parra and Windle²⁸) might be useful in supplying information about the percentage of DNA that is really present after the DNA amplification.

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SUMMARY

Human cytogenetics is the discipline that is directed towards the study of human chromosomes at the microscopic level. During cell division the DNA present in the nucleus of the cell is packaged in chromosomes which can be individually identified. After treatment with trypsin and staining with Giemsa the chromosomes display a so called GTG-banding pattern. This technique allows to distinguish 350-550 bands in a haploid set of chromosomes. The banding pattern is characteristic for each pair of chromosomes and thus allows the detection of aberrant chromosomes. Chromosome aberrations may be numerical (a number other than 46) or structural. In the group of structural aberrations that can be diagnosed, chromosomal material may be lost (deletion) or may be present twice (duplication). Furthermore, a part of a chromosome can be inverted within that chromosome (inversion) and chromosome parts can be exchanged between two or more chromosomes (translocation).

In the past 15 years fluorescence *in situ* hybridization (FISH) has become increasingly important in human cytogenetics. This technique can provide information about the presence and expression of specific DNA sequences in dividing and non-dividing cells. For FISH, a probe (a short DNA sequence) is labeled with a reporter molecule. This probe is hybridized with cellular DNA and binds with the complementary sequence within that DNA (hybridization). The hybridization site is visualized with a fluorescent molecule coupled with an antibody directed against the reporter molecule.

Despite GTG-banding and FISH there still exist chromosome aberrations that cannot be characterized completely. Micro-FISH is a technique that solves this problem. Microdissection combined with FISH (micro-FISH) comprises 4 different steps: (1) dissection of a (part of a) chromosome with microneedles, (2) amplification of the chromosomal material using the polymerase chain reaction (PCR), (3) labeling of the PCR product with a reporter molecule, and last (4) hybridization of the DNA on a normal metaphase and immunological detection. This procedure results in a fluorescent spot on the homologous chromosomes in the metaphase from which the dissected material originates.

In chapter 1, the development and the current status of micro-FISH, and its applications in molecular genetics and clinical cytogenetics are discussed. In chapter 2, a simple and efficient method for microdissection and DNA amplification is described. In chapter 3, micro-FISH was used to disclose the chromosomal origin of three marker chromosomes and the results were confirmed with chromosome painting. Chapter 4 reports on the application of micro-FISH to characterize a complex chromosome translocation. FISH with chromosome specific paints showed that at least four breakpoints were involved in this chromosome rearrangement, with micro-FISH a fifth breakpoint was detected which was characterized further with band-specific probes. Chapter 5 describes the application of micro-FISH for the detection of chromosomal deletions and the delineation of deletions in aberrant chromosomes. In chapter 6 the study of deletion detection was extended, a deleted region was characterized further with band-specific probes and the patient's phenotype is compared with other patients reported in the literature. Chapter 7 describes the application of a band-specific paint, generated by microdissection and subsequent DOP-PCR amplification, for the charac-

terization of a intrachromosomal duplication. Furthermore the clinical picture of the patient was compared with other patients reported in the literature with a duplication of chromosome region 15q11-q13. Chapter 8 contains a general discussion with conclusions and future perspectives.

In clinical cytogenetics, micro-FISH is a suitable technique to characterize chromosome aberrations (marker chromosomes, unbalanced translocation, duplications), especially when GTG-banding alone offers no solution. Also, loss off chromosomal material can be visualized and breakpoints can be determined more precisely. As only a few copies of chromosome (part)s under study have to be available for microdissection, micro-FISH is also suitable when only few mitotic divisions are available as is frequently the case in tumor material.

SAMENVATTING

De humane cytogenetica houdt zich bezig met het onderzoek van chromosomen van de mens op microscopisch niveau. Tijdens de celdeling of mitose, is het DNA aanwezig in de vorm van individueel te onderscheiden chromosomen. Na behandeling met het enzym trypsine en kleuring met de kleurstof Giemsa vertonen chromosomen een z.g. GTG-banderingspatroon. Per haploide set chromosomen kunnen zo 350-550 banden van elkaar onderscheiden worden. Dit banderingspatroon is karakteristiek voor elk chromosoompaar en stelt ons in staat afwijkende chromosomen op te sporen.

Chromosomale afwijkingen kunnen numeriek zijn (dus meer of minder dan het normale aantal van 46) of structureel. Bij de structurele afwijkingen kan er sprake zijn van verlies van een deel van het chromosoom (deletie) of van verdubbeling van een chromosoomdeel (duplicatie). Verder kan een deel van het chromosoom omgekeerd binnen een chromosoom aanwezig zijn (inversie). Ook kunnen delen van chromosomen onderling uitgewisseld zijn (translocatie).

Gedurende de afgelopen 15 jaar is fluorescente *in situ* hybridisatie (FISH) een steeds belangrijkere rol gaan spelen in het chromosomen onderzoek van de mens. Deze techniek kan informatie verschaffen over het aanwezig zijn en de expressie van specifieke DNA sequenties in delende en niet-delende cellen. Bij FISH wordt een probe (een klein DNA deeltje) gemerkt met een reporter molecuul. Deze probe wordt gebracht op cellulair DNA waarbij de probe bindt aan de complementaire sequentie in dat DNA (hybridisatie). Vervolgens wordt een antilichaam (met daaraan gekoppeld een fluorescerende stof) gericht tegen het reporter molecuul gebruikt om de plaats van de probe op het DNA zichtbaar te maken. Met behulp van een fluorescentie microscoop is de lokalisatie van de probe op het cellulair DNA te zien als een fluorescerend signaal.

Ondanks de beschikbaarheid van deze technieken zijn er echter nog steeds chromosomale afwijkingen die niet (volledig) gekarakteriseerd kunnen worden. Hiervoor is de afgelopen jaren micro-FISH ontwikkeld. Microdissectie gecombineerd met fluorescente *in situ* hybridisatie (micro-FISH) is een techniek waarbij een viertal verschillende stappen zijn te onderscheiden: (1) dissectie van een (deel van een) chromosoom m.b.v. een zeer dunne glazen naald, (2) vermeerdering van het gedissecteerd chromosomale materiaal met behulp van de polymerase ketting reactie, (3) inbouwen van een reporter molecuul in dat chromosomaal DNA, en tenslotte (4) hybridisatie van het gesynthetiseerde DNA op een normale metafase en detectie. Het resultaat van deze procedure is dat er in de metafase een fluorescentie signaal zichtbaar is op het chromosoom waarvan het gedissecteerd materiaal afkomstig is. Als er dus een stukje chromosoom van onbekende herkomst wordt gedissecteerd kan men na micro-FISH aan de hand van het fluorescentie signaal op een of meer chromosomen in een metafase bepalen van welk chromosoom dit materiaal oorspronkelijk afkomstig was. In hoofdstuk 1 van dit proefschrift is de ontwikkeling van micro-FISH beschreven, alsmede de stand van zaken op dit moment. Verder wordt gerapporteerd over de toepassing van micro-FISH in moleculair genetisch en klinisch cytogenetisch onderzoek. In hoofdstuk 2 wordt een eenvoudige maar efficiënte methode beschreven voor microdissectie en DNA amplificatie. In hoofdstuk 3 wordt micro-FISH gebruikt om de

herkomst van een drietal marker chromosomen te bepalen, waarbij de onderzoeksresultaten zijn bevestigd met chromosoom-specifieke paints. In hoofdstuk 4 is de karakterisering van een complexe chromosoom translocatie beschreven. FISH met chromosoom-specifieke paints toonde aan dat bij deze chromosomale afwijking ten minste 4 breukpunten aanwezig waren; met micro-FISH werd een 5de breukpunt aangetoond dat vervolgens met band-specifieke probes verder werd geanalyseerd. Hoofdstuk 5 beschrijft hoe met micro-FISH de positie van een deletie in een chromosoom en dus ook de breukpunten kunnen worden bepaald. Voorts werd aangetoond dat ook microdeleties zichtbaar kunnen worden gemaakt als de gegenereerde probe wordt gehybridiseerd op high-resolution chromosomen. In hoofdstuk 6 is een deletie die met micro-FISH werd aangetoond, met band specifieke probes nader in kaart gebracht. Dit gaf vervolgens de mogelijkheid de klinische kenmerken van het patiëntje in kwestie te vergelijken met in de literatuur beschreven patiënten. Een intrachromosomale duplicatie wordt in hoofdstuk 7 gekarakteriseerd, waarna een beschrijving volgt van andere patiënten uit de literatuur met een dergelijke duplicatie. Hoofdstuk 8 bevat een algemeen overzicht met conclusies en ontwikkelingen in de nabije toekomst. In de klinische cytogenetica is micro-FISH een geschikte techniek om chromosomale afwijkingen (marker chromosomen, ongebalanceerde translocaties, duplicaties) te karakteriseren, vooral als de klassieke GTG-banding te kort schiet. Ook kan met deze techniek verlies van chromosomaal materiaal worden aangetoond en kunnen breukpunten nader worden bepaald. Het feit dat slechts een klein aantal afwijkende chromosomen beschikbaar hoeft te zijn voor microdissectie, maakt dat deze techniek ook bruikbaar is als er maar geringe hoeveelheden cellen beschikbaar zijn, hetgeen regelmatig het geval is bij materiaal afkomstig van tumoren.

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A handwritten signature in black ink, appearing to be 'Joep', with a horizontal line underneath.

CURRICULUM VITAE

Johan Joseph Maria Engelen werd op 21 april 1953 geboren in de gemeente Nieuwenhagen. In 1971 behaalde hij het Atheneum-B diploma op het Bernardinus college te Heerlen.

Het kandidaatsexamen biologie werd behaald aan de Katholieke Universiteit Nijmegen in februari 1974, het doctoraalexamen in juni 1977. Afstudeervakken waren: Geobotanie (Prof. Dr. Westhof), Aquatisch Oecologie (Prof. Dr. den Hartog) en Medische Parasitologie (Prof. Dr. Meuwissen). Gedurende de studie werden student assistentschappen vervuld bij de afdelingen botanie en aquatische oecologie. Daarnaast werd de eerstegraads onderwijsbevoegdheid biologie gehaald. Na het afstuderen werkte hij enige maanden als leraar biologie bij het Collegium Marianum te Venlo.

In september 1978 begon hij met de studie Geneeskunde aan de Katholieke Universiteit Nijmegen. In augustus 1980 behaalde hij het kandidaatsexamen. Het Vrij doctoraal examen in deze studierichting werd behaald in oktober 1984 met als afstudeervakken Hematologie (Prof. Dr. Haanen), Epidemiologie (Prof. Dr. Sturmans) en Toxicologie (Prof. Dr. Henderson, Dr. Scheres). Van 16 juni 1982 tot 1 april 1984 was hij als wetenschappelijk medewerker verbonden aan het Instituut voor Antropogenetica van de Katholieke Universiteit te Nijmegen.

Na het afstuderen werkte hij voor NOTOX Safety & Environmental Research B.V. in Den Bosch, was enige maanden werkzaam als leraar biologie aan het St. Maartens College te Maastricht en trad op 1 juni 1986 in dienst van de Rijksuniversiteit Limburg te Maastricht bij de capaciteitsgroep Arbeidsgeneeskunde. Sinds 1 augustus 1988 is hij als universitair docent werkzaam bij de vakgroep Moleculaire Celbiologie & Genetica van de Rijksuniversiteit Maastricht en gedetacheerd bij de Stichting Klinische Genetica Limburg.

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