INTRODUCTION

Fluorescent in situ hybridization is a powerful method that permits visual identification of specific nucleic acid sequences in metaphase or interphase cells (Trask, 1991; Pinkel et al., 1986). This technique is based on hybridization of a labeled nucleic acid probe to complementary target sequences on fixed biological materials such as whole-tissue sections, cells, or morphologically preserved chromosomes (Tongiorgi et al., 1998). After hybridization, the newly formed hybrids can be detected on a fluorescent microscope (Leitch et al., 1994).

In addition to routine cytogenetic analysis, where karyotyping is the most conclusive test for chromosome analysis, FISH technique has been introduced in order to achieve a higher sensitivity, specificity, and resolution of chromosomes (Trask, 1991). These characteristics, together with other advantages such as speed and reliability, make FISH available to any laboratory with a standard fluorescent microscope and applicable in clinical cytogenetics, prenatal diagnosis, diagnosis of infectious diseases, and tumor biology (Laudy, 1990; Liu et al., 1993).

The accuracy, reproducibility, and reliability of FISH analysis depend mostly on the specificity and sensitivity of the probes used and the hybridization detection efficiency (Divane et al., 1994). The probes can be labeled either with a fluorescent tag (direct method) or with non-fluorescent molecules which are then detected by fluorescently labeled antibodies (indirect method) (Leitch et al., 1994). There are many different types of DNA probes that can be used in FISH, including: CEP (chromosome enumeration probes), LSI (locus specific identifier probes), telomere-specific DNA probes, WCP (whole chromosome painting probes), unique sequence microdeletion probes, and unique sequence translocation junction probes.

One of the special applications of FISH has been the use of whole chromosome probes, known as chromosome painting. The WCP is a collection of DNA fragments that hybridize to different sequences along the entire length of the chromosome (Strachan and Read, 1996). Chromosome painting has found increasing application in identification of complex chromosome rearrangements, including de novo rearrangements, and small marker chromosomes, particularly in clinical and cancer
cytogenetics (Strachan and Read, 1996).

Alu sequences are classified as short interspersed nuclear elements (SINEs) and represent the largest family of mobile elements in the human genome (Batzer and Deininger, 2002). Approximately 900,000 copies of this 300 base pairs long sequence are distributed throughout the human genome, giving an average distance between copies of 4 kilobases (Nelson et al., 1989). They are usually found in introns, 3' untranslated regions of genes and intergenic genomic regions (Batzer and Deininger, 2002). The Alu repeat is primate specific, but homologous repetitive elements have been found in other mammals too (Strachan and Read, 1996). However, there is sufficient sequence divergence to reduce cross hybridization, of human and rodent Alu repeats, for example (Nelson et al., 1989).

Kariya et al. (1987) defined the conserved and variable regions within the human Alu sequences. This served as the basis for design of the inter-Alu-PCR primers used to direct PCR synthesis toward the next upstream and downstream Alus with the intent to amplify regions between Alu segments (Liuet al., 1993). The given approach served as a basis for extraction of human DNA from interspecific somatic cell hybrids, which permitted production of chromosome- and chromosome region-specific probes for FISH (Liuet al., 1993).

Somatic cell hybrids can be generated by fusion of cultured cells from different species (Strachan and Read, 1996). For the purpose of human genetic mapping, hybrid cells are typically constructed by fusing human and rodent cells (usually mouse or hamster). The hybrid cells are initially unstable and for reasons that remain unknown, most human chromosomes are lost in subsequent rounds of cell division (Strachan and Read, 1996). This gives rise to a variety of relatively stable hybrid cell lines, each with the full set of rodent chromosomes plus a few human chromosomes, the simplest one containing a single donor chromosome (monochromosomal hybrids). The loss of the human chromosomes occurs essentially at random, but can be controlled by selection.

Here we present a version of the Alu-PCR method modified for production of WCP 19 using monochromosomal cell hybrids. In setting up the conditions for Alu-PCR, we created a basis for the generation of whole chromosome-specific probes from available monochromosomal cell hybrids. By doing so, we established a cheap and fast approach to generation of FISH probes particularly useful for unambiguous identification of complex chromosomal rearrangements associated with cancer.

MATERIALS AND METHODS

Cytogenetic study

A bone marrow sample from a male diagnosed with acute myeloid leukemia (AML) was analyzed. Cytogenetic analysis was performed both on unstimulated cells by direct preparation and on cells following 24 h cultivation in RPMI 1640 medium containing 25% fetal calf serum at 37°C. Peripheral blood of a female was used as a control. Leukocytes were cultured for 72 h in RPMI 1640 medium containing 25% fetal calf serum at 37°C, with phytohemagglutinin stimulation.

Chromosomes were examined by the modified Giemsa stain technique described previously as HG-banding (Novak et al., 1994). The karyotype was arrayed and described according to the International System for Human Cytogenetic Nomenclature 2005 (Shaffer and Tommerup, 2005). Clones were defined as two or more metaphases with the same structural abnormality or the same extra chromosome, or as three or more metaphases that lacked the same chromosome.

Alu-PCR

DNA from monochromosomal cell hybrid GM10612 (HGMP-RC, Cambridge, UK) containing human chromosome 19 on only hamster background was used as a template for Alu-PCR reaction.

The Alu-PCR was performed to generate a painting probe for chromosome 19. Alu-specific oligonucleotide primers ALE1 (GCCCTCCAAAAGTCTGGGATTACAG) and ALE3 (CCACTGCACTCCAGCCTGGG) were designed from the published sequence (Cole et al., 1991). The PCR reaction took place in 30 μl of a reaction medium composed of 100 ng GM10612 DNA, 1.3 μM of both oligonucleotide primers, 2.5 mM of each dNTP, reaction buffer (50 mM KCl; 10 mM Tris-HCl, pH 9.0; 0.1% Triton X 100) (Promega), 7.6 mM MgCl2, BSA (170 μg/ml), 10 mM β-mercaptoethanol, and 1.25U Taq polymerase. Following initial denaturation at 94°C for 1 min, 35 cycles of PCR were carried out with denaturation at 94°C for 30 sec, annealing at 63°C for 1 min, and elongation at 72°C for 7 min. The PCR product was visualized with ethidium bromide on 1% agarose gel.
**Probe labeling**

The probe for chromosome 19 prepared by Alu-PCR was labeled by nick translation with SpectrumGreen dUTP (Vysis).

**Hybridization and detection**

The labeled probe for chromosome 19 (100 ng) was combined with 1 μg of COT-1 DNA and precipitated. COT-1 DNA is highly enriched with repetitive sequences and has been used to block cross-hybridization of chromosome painting probes with human repetitive DNA sequences of the AluI and KpnI families (Newkirk et al., 2005; Lengauer et al., 1990). The precipitated probe and COT-1 DNA were dissolved in 16 μl of a hybridization buffer consisting of 50% formamide, 10% dextran sulfate, 1% SDS, 1XDenhardt’s, 2XSSC, and 0.04 M sodium phosphate, pH 7.0.

Hybridization and detection of labeled probes was performed as recommended by the Nick Translation Kit (Vysis) with some modifications. Briefly, slides were immersed in denaturation solution (70% formamide/2xSSC) for 5 minutes at 75°C, dehydrated through an ethanol series (70%, 85%, and 100% ethanol, 1 min each), and air-dried. The probe was denatured for 5 min in 73°C, cooled on ice, and applied to the slide, sealed under a coverslip with a rubber cement, and incubated in a humidified box at 37°C overnight. Following hybridization, the coverslips were removed by rinsing in 0.4xSSC/0.3% NP-40 at room temperature. Slides were washed in 0.4xSSC/0.3% NP-40 at 73±1°C for 2 min, then washed in 2xSSC/0.1% NP-40 for 30 sec at room temperature and air-dried in darkness.

The slides were mounted in 0.4 mg/mL DAPI (4',6-diamidino-phenylindole) counterstain in Vectashield Antifade Buffer, viewed under an Olympus BX51 fluorescent microscope with appropriate filters for detection of SpectrumGreen and DAPI, and analyzed using Cytovision 3.1 software (Applied Imaging Corp.).

**RESULTS AND DISCUSSION**

In order to apply FISH for rapid detection of chromosomal translocations, we used the method of chromosome painting. The involvement of chromosome 19 in many types of malignancies prompted us to seek a method based on Alu-PCR for generation of a chromosome 19-specific probe that could be used for FISH analysis. The first step was to establish conditions for Alu-PCR using as a template DNA from a chromosome 19 monochromosomal somatic cell hybrid (GM10612).

Kariya et al. (1987) compared nucleotide sequences of 50 human Alu repeats and concluded that Alu conserved regions consist of a 25-bp region between nt positions 23 and 47 and a 16-bp region between nt positions 245 and 260. Based on this finding, we used primers (ALE1 and ALE3) that anneal to defined conserved elements of the human Alu repeats in order to amplify the region between Alu sequences (Cole et al., 1991). We modified the Alu-PCR procedure presented by Cole et al. (1991) as described in the Materials and Methods section in this paper. As expected, we obtained a smear of DNA (ranging from 0.4 to 12 kb) as a result of inter-Alu amplification (Fig. 1, lane 3). The fact that total human DNA amplifies as a smear suggests that sufficient numbers of fragments are produced to enable visualization of the targeted chromosome 19. As was mentioned earlier, this approach makes it possible to selectively amplify human DNA from only somatic cell hybrids.

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**Fig. 1.** Agarose gel electrophoretic separation of Alu-PCR products obtained by amplification of human DNA from somatic cell hybrid GM10612. Lane 1. 1 Kb DNA Ladder (GibcoBRL). Lane 2. PCR control reaction (no DNA). Lane 3. Products of PCR reaction using ALE1 and ALE3 primers are visible as a smear ranging from 0.4 to 12 Kb.
We then labeled the amplified fragments with SpectrumGreen and continued our study by performing FISH with the obtained painting probe specific for chromosome 19.

To check quality of the generated WCP 19, FISH was first performed on a control sample. To be specific, we used chromosome spreads obtained from a female patient for whom classical cytogenetical analysis (HG-banding technique) revealed the normal 46,XX karyotype (Fig. 2). As shown in two metaphases on Fig. 3, we detected green signals on each chromosome 19 as a result of specific hybridization of the generated painting probe. Note that the centromere regions remain un-painted. This could be attributed either to a lack of Alu sequences in this region or to their under-representation (Moyzis et al., 1989). A substantial body of published indicates at involvement of chromosome 19 in different chromosome rearrangements related to pathological conditions. Translocation (1;19) is usually linked to acute lymphoblastic leukemia (Privitera et al., 1992; Tchinda et al., 2002; Mazieres et al., 2005; Piccaluga et al., 2006). Pre-B-ALL are generally associated with t(1;19) and (17;19), while t(7;19) is associated with T lineage ALL (Heim and Miteff, 1995). Also, t(1;19) was found in patients with myeloid neoplasias (Tchinda et al., 2002). Translocation (15;19) was detected in a female with thymic carcinoma (Kubonishi et al., 1991) and

Fig. 2. Cytogenetical analysis of control sample. Using HG-banding technique the normal karyotype: 46,XX has been revealed.

Fig. 3. FISH analysis of control sample with WCP 19. Chromosomes were stained with DAPI (blue). The painting probe prepared by Alu-PCR specifically labeled both arms of chromosome 19 (green, indicated by arrows).
t(14;19) in lymphoproliferative disorders (Robinson et al., 2004). Cytogenetic analysis revealed t(5;19) in a thyroid follicular adenoma (Roque et al., 1992). Also, translocation (11;19) has been found in mucoepidermoid carcinoma of the salivary gland (Nordkvist et al., 1994) and in leukemia cases, both ALL and AML (Huret et al., 1993; Rubnitz et al., 1996).

Accordingly, we applied the obtained WCP 19 for analysis of a patient diagnosed with acute myeloid leukemia (AML).

Results of cytogenetic analysis obtained by the HG-banding technique, showed the following: 47,XY,t(1;19)(p13;?),+der(1)t(1;19)(p13;?)\[22]/46,XY\[1], e.g., a complex karyotype carrying the balanced translocation (1;19) and one additional derivative chromosome. The karyogram of this patient is presented in Fig. 4.

In order to confirm the cytogenetic results, FISH was performed on chromosome spreads from the same patient using WCP 19 (Fig. 5). The FISH demonstrated the presence of one normal chromosome 19 and signals

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**Fig. 4.** Cytogenetical analysis of sample from patient with AML. Karyotype of abnormal cell clone: 47,XY,t(1;19)(p13;?),+der(1)t(1;19)(p13;?) is shown. Translocated chromosomes (1;19) and the derivative chromosome are indicated by arrows.

**Fig. 5.** FISH analysis of sample from patient with AML using WCP 19. Chromosomes are labeled with DAPI (blue), and chromosome 19-specific sequences are labeled in green. Chromosome 19, translocated chromosomes (1;19), and the derivative chromosome are indicated by arrows.
on three other chromosomes, two of which were previously characterized cytogenetically as translocated (1;19) and the third characterized as an additional derivative chromosome. Thus, we verified that the translocation involved chromosome 19 and also that the additional derivative chromosome originated from a duplication of a product of balanced translocation.

In conclusion, we modified the Alu-PCR method for generation of a whole chromosome painting probe specific for human chromosome 19. This was done in order to confirm results of cytogenetic analysis on a patient with a complex karyotype carrying the balanced translocation (1;19) and an additional derivative chromosome. By employing FISH with the generated painting probe, we confirmed that the translocation involved chromosome 19 and that the additional derivative chromosome also contained parts of chromosome 19. Further application of WCP 19 and generation of painting probes for other human chromosomes or parts of chromosomes using Alu-PCR and somatic cell hybrids should facilitate the analysis of complex chromosome rearrangements in clinical and cancer cytogenetics in our country.

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REFERENCES


СИНТЕЗА ПРОБЕ ЗА БОЈЕЊЕ ХРОМОЗОМА КОРИШЋЕЊЕМ МОНОХРОМОЗОМАЛНИХ ЉЕЛИЈСКИХ ХИБРИДА ЈА Алу-РЕАКЦИЈЕ ЛАНЧАНОГ УМНОЖАВАЊА

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Метода флуоресцентне in situ хибридизације (енгл. FISH) је нашла широку примену у бицинауци и дијагностици. Бојење хромозома (енгл. chromosome painting) представља специјалну варијанту ове методе која се користи за идентификовање сложених хромозомских реаранжмана. У овом раду смо приказали модификовану верзију Alu-реакције ланчаног умноожавања (Alu-PCR) коју смо применили за синтезу пробе специфичне за хумани хромозом 19 коришћењем монохромозомалног ћелијског хибрида. Успостављена метода представља брз и јефтин начин за синтезу проба специфичних и за остале хумане хромозоме. Ова методологија је изузетно корисна за претцизну идентификацију сложених хромозомских реаранжмана који најчешће прате разне малине трансформације.