RAPID COMMUNICATION

Direct Visualization of Dispersed 11q13 Chromosomal Translocations in Mantle Cell Lymphoma by Multicolor DNA Fiber Fluorescence In Situ Hybridization

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Sev eral hematologic malignancies are associated with specific chromosomal translocations. Because of the dispersed distribution, chromosomal breakpoints may be difficult to detect using molecular techniques. We present a new application of a recently developed method, DNA fiber fluorescence in situ hybridization (fiber FISH), which allows direct visualization and mapping of chromosomal breakpoints. We tested this method for detection of the t(11;14)(q13;q32) translocation in mantle cell lymphoma. In DNA fiber FISH, a series of fluorochrome-labeled DNA probes covering several hundreds of kilobasepairs is hybridized to linear DNA molecules (or fibers) prepared from frozen tissue or intact cells. By using alternate fluorescent colors, a potential breakpoint region is stained in a color barcode pattern. Breaks in this region will split the barcode in two complementary parts, from which the breakpoint position can be derived. We used a 250-kb barcode covering the BCL-1 locus to detect 11q13 breakpoints in 20 well-characterized mantle cell lymphomas. A t(11;14) was shown by cohybridization of these probes with probes for the Ig heavy chain locus at 14q32. In 18 of 20 mantle cell lymphomas, a breakpoint within the 11q13/BCL-1 barcode was shown by the presence of multiple, complementary translocation products. Fusion of 11q13 and 14q32 sequences on single fibers indicating t(11;14)(q13;q32) was found in all 18 breakpoint-positive mantle cell lymphomas. In one additional case, fusion of an intact 11q13 barcode with 14q32 sequences indicated a breakpoint 100 kb centromeric of the major translocation cluster of BCL-1. Within the 120-kb region of BCL-1, breakpoints were widely scattered. This explains why, so far, a BCL-1 breakpoint had been detected by Southern blot analysis in only 10 of 19 cases. DNA fiber FISH analysis showed a t(11;14) in 95% of mantle cell lymphoma. The results indicate that DNA fiber FISH is a rapid, simple, and equally powerful method for detection of clustered and dispersed translocation breakpoints.

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stant regions of the Ig heavy chain gene complex at 14q32. Nineteen of 20 mantle cell lymphomas had previously been analyzed by Southern blot using all available probes, detecting an 11q13 breakpoint in 10 cases. Our current findings indicate that DNA fiber FISH is superior to Southern blot analysis in detecting chromosomal breakpoints.

**MATERIALS AND METHODS**

*Tissue samples.* Frozen tissue of 18 mantle cell lymphomas, 3 follicular lymphomas [1 with a documented t(14;18) translocation], 2 lymphoblastic lymphomas, 1 gastric low-grade lymphoma of the MALT, and 2 normal tonsils were obtained from the tissue bank of the Department of Pathology of the University of Leiden (Leiden, The Netherlands). Additionally, viable frozen tumor cells of 2 mantle cell lymphomas were obtained from the Department of Hematology of the University of Leiden. In 1 mantle cell lymphoma (p 638), banding analysis was performed and showed a t(11;14)(q13;q32) (Dr G.C. Beverstock, Department of Human Genetics, University of Leiden). Immunohistochemistry and immunofluorescence on frozen tissue sections or suspended cells of mantle cell lymphomas showed a typical CD19, CD22, and CD5 expression pattern as well as strong expression of IgM but not of CD10. All cases showed overexpression of the *cyclin D1* gene, as previously demonstrated by Northern blots. Western blots, and/or immunohistochemistry on paraffin sections using the DCS6 monoclonal antibody. Frozen material of 19 of 20 tumors had previously been analyzed by Southern blot analysis for rearrangement within the BCL-1 locus using 5 different plasmid probes. The human foreskin fibroblast cell line VH25 was provided by the Department of Radiation Genetics of the University of Leiden.

*DNA fiber preparation.* Preparations of DNA fibers were made according to the halo technique, with some modifications. Forty-micrometer frozen tissue sections were suspended in cold phosphate-buffered saline (PBS). The resulting nuclear suspension was applied to object slides and nuclei were allowed to adhere for 2 minutes. Slides were dipped for 30 seconds in the following ice-cold solutions: solution 1 (25 mmol/L Tris-HCl, pH 8.0, 10 mmol/L MgCl₂, 0.5 mmol/L CaCl₂, and 0.5% Nonidet NP-40), solution 2 (25 mmol/L Tris-HCl, pH 8.0, 0.2 mmol/L MgCl₂, and 2 mol/L NaCl), and solution 3 (solution 2 + 40 μg/mL propidium iodide). Slides were irradiated with UV light (254 nm, 7,000 μW/cm²) for 7 minutes, dipped in ice-cold solution 4 (25 mmol/L Tris-HCl, pH 8.0, 0.2 mmol/L MgCl₂, and 0.2 mol/L NaCl) and solution 5 (25 mmol/L Tris-HCl, pH 8.0, and 0.2 mmol/L MgCl₂), dipped twice in H₂O, and air-dried.

*Probes and in situ hybridization.* The cosmids cos6.22, cos3.62, cos3.91, cos6.7, and cosH1.5 were isolated from a cosmid library (ICRF Laboratories, London, UK). The cos6.22, cos3.62, cos3.91, and cos6.7 were isolated from a cosmid library (ICRF Laboratories, London, UK). Immunohistochemistry and immunofluorescence on frozen tissue sections or suspended cells of mantle cell lymphomas showed a typical CD19, CD22, and CD5 expression pattern as well as strong expression of IgM but not of CD10. All cases showed overexpression of the *cyclin D1* gene, as previously demonstrated by Northern blots. Western blots, and/or immunohistochemistry on paraffin sections using the DCS6 monoclonal antibody. Frozen material of 19 of 20 tumors had previously been analyzed by Southern blot analysis for rearrangement within the BCL-1 locus using 5 different plasmid probes. The human foreskin fibroblast cell line VH25 was provided by the Department of Radiation Genetics of the University of Leiden.

RESULTS

The 3 cosmid and 2 PI probes for 11q13 were hybridized to DNA fiber preparations of fibroblast cell line VH25. On the basis of the hybridization pattern, a map of these 5 clones was constructed and a 250-kb color barcode was generated. Next, the 11q13 probes were hybridized to DNA fiber preparations of 20 mantle cell lymphomas, 6 other non-Hodgkin’s lymphomas, and 2 normal tonsils. In 18 of 20 mantle cell lymphomas, broken barcodes were observed in addition to the intact germline barcode. Each of these 18 mantle cell lymphomas contained two different but complementary broken barcodes, probably representing the 11q- and 14q+ translocation products. In all cases, more than 10 identical 11q- products and 10 identical 14q+ products complementary to the 11q- products were easily found. An example is shown in Fig 1. Each case, five of the complete and five of each of both broken barcodes were measured. Figure 2 shows a composite image of barcodes found in one tumor. The mean breakpoint position in each tumor was calculated and is shown in the map of Fig 3. All 8 negative controls and the 2 negative mantle cell lymphomas showed only very rare and randomly distributed breaks that may have been generated during preparation of DNA fibers.

To determine whether the translocation partner of chromosome 11q14 is the Ig heavy chain gene region at 14q32, all tumors were also hybridized with a combination of multiple 11q13 and 14q32 probes (a 14q32 barcode on normal DNA fibers of the VH25 cell line was constructed by use of multi-
DETECTION OF TRANSLOCATIONS BY FIBER FISH

**Fig 1.** Fiber FISH to detect t(11;14) in mantle cell lymphoma. Three different barcode patterns found in DNA fiber preparations of mantle cell lymphoma case p67 hybridized with the 11q13 probes. Cosmid and P1 probes are indicated below each panel (see also Fig 3 for the 11q13/BCL-1 region). The upper panel, marked 11q13, shows hybridization on a normal chromosome 11. The P1 probe B1587 and cosmid probes cos 6.22 and cos 3.91 were labeled with biotin and visualized in red with Texas red. The P1 J0777 and cosmid cos 3.62 were labeled with digoxigenin and visualized in green with fluorescein isothiocyanate. Overlap of B1587 and J0777 results into a yellow color. The second (marked 14q+) and third (marked 11q-) panels show the complementary patterns representing the 14q+ and 11q− translocation products, respectively. The breakpoint is within the region covered by the J0777 probe (arrows). The fourth panel shows a separate experiment to demonstrate colocalization of 11q13 and 14q32 signals on a single DNA fiber indicating t(11;14). A fragment of the P1 J0777 (green) from 11q13 is juxtaposed to a small part of cosmid U2-2 (covering part of Cμ, Cµ, and the JH gene segments; in red), cos 3/64 (covering C6, Cµ, Sµ, and JH gene segments; in green), and pb14MD (covering a part of the intron between the Ig Cα and Cγ3 gene; in red), all derived from 14q32. Cosmids Ig6 and Ig9 show complex hybridization patterns, probably due to evolutionary duplication and strong homologies within the Ig locus.
ple cosmid and plasmid probes; J.W.V., in preparation). Fusion products of the 11q13 and 14q32 barcodes were observed in 18 of 18 breakpoint-positive mantle cell lymphomas (an example is shown in Fig 1). Additionally, a fusion product was found in one of the two negative tumors, with a large gap between the intact 11q13 barcode and the 14q32 barcode. Measurement of this gap enabled us to map the breakpoint 78 kb centromeric from the 5' end of B1587 and approximately 100 kb from the MTC.

In 1 mantle cell lymphoma (p001), no breakpoint could be detected. To find a possible breakpoint in an even wider region around the cyclin D1 gene at 11q13, we investigated this case with interphase FISH on nuclei isolated from frozen tissue sections using two different cosmids pairs in total.

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**Fig. 2.** 11q13 breakpoint analysis by fiber FISH. Computer-processed composite image of 5 normal (upper panel), 5 11q- (middle panel), and 5 14q+ (lower panel) hybridization patterns, as found in mantle cell lymphoma. All images were randomly chosen and photographed. Differences in degree of DNA condensation on glass slides were eliminated by horizontal stretching or compressing of the images and normalization to the total length of the barcode.

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**Fig. 3.** Mapping of 11q13 breakpoints in 19 mantle cell lymphomas. Map of the P1 and cosmid clones (open bars) in the 11q13 probe mix used for fiber FISH detection of breakpoints in mantle cell lymphoma. The position of the plasmid probes BCL1-1b, p94PS, p11EH, and BB4 has been determined by restriction mapping.11,15,20 These probes that have been used for Southern blot analysis of the present series12,20 are shown. The black horizontal bars indicate the detection area of each probe when used in combination with the restriction enzymes EcoRI, HindIII, and BamHI. The 19 breakpoints mapped by fiber FISH are indicated below. The 10 breakpoints that had also been detected by Southern blot analysis are underlined. In case p215, no Southern blot analysis could be performed. A detailed map of the whole 11q13 region will be published separately (J.W.V., in preparation).
spanning a region of approximately 1,200 kb around the cyclin D1 gene at 11q13. Sixty percent of interphase nuclei showed a single set of cohybridizing spots, but no breakpoints within this part of 11q13. Additional hybridization with a probe specific for centromeric DNA of chromosome 11 showed single spots in a majority of cells, suggesting monosomy 11 (data not shown).

To compare the localization of the breakpoints as determined by DNA fiber FISH with the Southern blot data, the positions of the plasmid probes used for Southern blot analysis and their detection range as defined by the appropriate restriction sites, were added to the fiber FISH map. Because the position of probe p11EH was not sufficiently documented, this probe was mapped by fiber FISH by cohybridization of this probe with the P1 probe J0777. Mapping of all 11q13 breakpoints by DNA fiber FISH showed that all 10 Southern blot-positive lymphomas had a breakpoint localized in the detection area of the respective probe and that all 9 Southern blot-negative tumors had a breakpoint outside of these areas (Fig 3). Additionally, the single case not tested by Southern blotting (case p215) contained an 11q13 breakpoint outside these areas.

DISCUSSION

We showed the usefulness of DNA fiber FISH for the detection and mapping of chromosomal translocations. Using this novel technique, we found a translocation t(11;14) in 95% of mantle cell lymphomas versus in 53% using conventional Southern blot analysis. Mapping of 11q13 breakpoints by DNA fiber FISH showed a complete concordance between this method and Southern blot analysis.

Many translocation breakpoints in lymphoid neoplasias, including mantle cell lymphoma, Burkitt's lymphoma, and follicular lymphoma, are dispersed over large areas of several hundreds of kilobasepairs or are clustered in widely separated regions. Using DNA fiber FISH, we were able to detect translocations in an area of 250 kb in a single experiment. Previous experiments have shown that larger areas up to 500 kb can be covered in a single hybridization (J.W.V., unpublished results).

Chromosomal breakpoints can be detected by various molecular and other techniques. In order of detection range, these techniques are metaphase cytogenetics (conventional banding analysis), interphase cytogenetics with FISH, pulsed field gel electrophoresis, Southern blot analysis, and PCR. Conventional banding analysis needs isolation and culturing of viably suspended tumor cells. Furthermore, the technique has a limited resolution and sensitivity. Techniques with a detection area comparable to that of fiber FISH are interphase FISH and pulsed field gel electrophoresis. What makes fiber FISH more attractive than interphase FISH is the ability to precisely map breakpoints and the unambiguousness of the results. Specific signals in fiber FISH are always recognized as fluorescent strings in alternating colors, which are easily distinguishable from the starry sky-like nonspecific signal. Therefore, hybridization and washing conditions are far less critical in fiber FISH than in interphase FISH and even enable the use of relatively small plasmid probes, as shown in the current investigation. Because of the specificity of the fluorescent pattern in fiber FISH, observation of relatively few complementary barcodes with identical breaks is sufficient to identify a chromosomal breakpoint. As shown, the specificity of breakpoint analysis can be enhanced by simultaneous visualization of the relevant juxtaposed chromosomal regions, in this case 11q13 and 14q32 barcodes, on single DNA fibers. Pulsed field gel electrophoresis is a relatively difficult method not routinely applicable for breakpoint analysis. As compared with fiber FISH, Southern blot analysis has a limited detection range (<20 to 25 kb) that depends on the available probes and restriction sites at the locus of interest. Thus, one single fiber FISH experiment enables screening of genomic areas that are 15 to 25 times the area covered by an average Southern blot analysis. Although PCR is a very fast method, it has a very small detection area of up to several kilobases, resulting in a high failure rate in cases of dispersed breakpoints. DNA fiber FISH may also be useful for the analysis of tumors with a fusion gene such as BCR/ABL in chronic myeloid leukemia, in which no RNA is available for reverse transcription-PCR.

Fiber FISH is a relatively rapid method. A tissue sample retrieved on the morning of day 1 can be diagnosed in the afternoon of day 2, provided that labeled probes are available. DNA fibers can be isolated from fresh material or archival frozen tissue and there is no need to make viable cell suspensions or to enrich for tumor cells. However, the sensitivity of the method for detection of limited numbers of tumor cells, for instance, in a setting of residual disease after therapy, has to be explored by dilution experiments.

Recently, we and others showed that cyclin D1 is overexpressed in many if not all mantle cell lymphomas. The present study shows that there is a near-absolute if not full correlation between overexpression and t(11;14)(q13;q32), indicating that, at the genetic level, mantle cell lymphoma is a relatively uniform disorder. In only 1 mantle cell lymphoma could an 11q13 breakpoint not be detected in the 250-kb region surrounding the cyclin D1 gene by fiber FISH or in an approximately 1.200-kb region as determined by interphase FISH analysis. In this tumor with loss of one chromosome 11, cyclin D1 overexpression may have been caused by a genetic or other defect that cannot be detected by FISH. For instance, in some mantle cell lymphomas, small deletions contributing to a reduced degradation of cyclin D1 mRNA have been described in the 3′ untranslated region of the gene.

The breakpoints mapped by fiber FISH were scattered over the entire area between the MTC and the cyclin D1 gene and one was 100 kb centromeric from the MTC. Apart from the MTC, a large proportion of breakpoints mapped around probe p11EH. We did not identify any breakpoint telomeric of the cyclin D1 gene, a region involved in rare variant translocations to Ig light chain genes.

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Direct visualization of dispersed 11q13 chromosomal translocations in mantle cell lymphoma by multicolor DNA fiber fluorescence in situ hybridization

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