Interphase fluorescence *in situ* hybridization assay for the detection of rearrangements of the *EVI-1* locus in chromosome band 3q26 in myeloid malignancies

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Background and Objectives. Rearrangements of the *EVI-1* locus in chromosome band 3q26 are associated with a poor prognosis in myeloid malignancies. To aid the diagnosis of such aberrations, and possibly disease monitoring, we established an interphase fluorescence *in situ* hybridization (FISH) assay for the affected breakpoint region.

Design and Methods. Several overlapping PAC (P1-derived artificial chromosome) clones centromeric to the *EVI-1* gene were labeled with a red fluorescent dye, and PAC clones telomeric to *EVI-1* with a green fluorochrome. This dual-color probe was hybridized to cytogenetic preparations of cell lines and patients' samples, which were also investigated for the presence of 3q26 rearrangements by chromosome banding analysis.

Results. In nuclei without 3q26 rearrangements, two pairs of co-localized red and green signals were observed, while separation of one red/green signal pair or splitting of one red or one green signal was found when 3q26 aberrations were present. The threshold value for true positivity, as determined on 20 samples from patients with myeloid malignancies without 3q26 rearrangements, was 10.2% for separation of one red/green signal pair, and 1% and 1.3% for splitting of one red or one green signal, respectively. In 17 samples from patients with a 3q26 aberration, the percentage of aberrant cells was significantly above these threshold levels.

Interpretation and Conclusions. We established an interphase FISH assay that efficiently identifies chromosome breakpoints affecting the *EVI-1* locus in 3q26, and represents a useful complement to chromosome banding analysis for the detection of such aberrations.

Key words: interphase FISH, 3q26, *EVI-1*, myeloid leukemia.

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Several recurrent chromosome rearrangements involving band 3q26 in patients with myeloid malignancies affect the zinc finger transcription factor oncogene *EVI-1* or its surrounding sequences. In cases with inv(3)(q21q26), the 3q26 breakpoints tend to be located downstream, that is, centromeric, of *EVI-1*, while 3q26 breaks associated with the translocation t(3;3)(q21;q26) have been reported to occur mostly upstream and telomeric of this gene. Altogether, the 3q26 breakpoints in these rearrangements are scattered over several hundred kilobases.[1-3] The inv(3) and the t(3;3) also share a 100kb breakpoint region in 3q21, and effect overexpression of the *EVI-1* gene, possibly due to its juxtaposition to enhancer sequences of the *Ribophorin I* gene in 3q21.[1,3-9] The translocations t(3;12)(q26;p13) and t(3;21)(q26;q22), on the other hand, lead to the formation of fusion transcripts involving *EVI-1*, and sometimes also the neighboring genes *EAP* and *MDS1*. The partner genes in 12p13 and 21q22 are *ETV6* and *AML1*, respectively.[10-12]

The above mentioned chromosome rearrangements may occur in almost all FAB types of acute myeloid leukemia (AML), as well as in chronic myeloid leukemia (CML) and myelodysplastic syndromes (MDS), and are correlated with a particularly aggressive course of disease and unfavorable treatment outcome.[13-18] Tools to confirm or establish their presence or absence at diagnosis, in particular in samples for which chromosome banding analysis is hampered by low quality or insufficient numbers of metaphase cells, and to monitor response to therapy or disease progression are, therefore, highly desirable. Interphase fluorescence *in situ* hybridization (interphase FISH) assays which serve these purposes have been established for a number of recurrent chromosome rearrangements, but, to the best of our knowledge, not for 3q26 aberrations. We therefore chose PAC clones centromeric and telomeric to the *EVI-1* gene, and established an interphase FISH assay that detects 3q26 rearrangements by the separation of normally co-localizing red and green signals.

Design and Methods

Patients' samples

Peripheral blood and bone marrow samples were submitted to our laboratories for diagnostic cytogenetic analysis. FISH assays were performed as part of these analyses, or retrospectively on archival samples from patients with 3q rearrangements. Threshold values for true positivity were determined on samples from 20

patients with myeloid malignancies without cytogenetically detectable 3q26 rearrangements (15 AML, 3 MDS, and 2 CML; 10 female, 10 male; median age, 60 years). All samples had been taken at the time of initial diagnosis, and had been submitted to the Institut fuer Medizinische Biologie in Vienna for cytogenetic analysis between May 2001 and February 2002. These samples were selected to represent various FAB types of AML, as well as CML and MDS.

Samples from 17 patients with 3q26 rearrangements (11 AML, one biphenotypic acute leukemia, one MDS, 4 CML; 10 female, 7 male; median age, 50 years) had been collected and diagnosed cytogenetically in our laboratories between 1991 and 2002. Three patients had a t(3;21)(q26;q22), 3 a t(3;3)(q21;q26), and 11 an inv(3)(q21q26).

Culture of established cell lines

All cell lines were cultured at 37°C in a 5% CO_2 atmosphere. JH cells carrying a t(3;3)(q21;q26), (RW and CF, unpublished results) (a gift from Drs V. Diehl and C. Scheid), HNT-34 cells carrying a t(3;3)(q21;q26),[19] (a gift from Dr H. Hamaguchi) and MPD cells with no 3q26 rearrangement[20] (a gift from Drs C. Paul and M. Baumann) were grown in RPMI 1640 medium supplemented with 10% FCS, and, for MPD cells, 1 mM sodium pyruvate. Mutz-3 cells carrying an inv(3) (q21q26)[21,22] (a gift from Drs H. Drexler and R. MacLeod) were grown in MEM-α with 20% FCS and 36 ng/mL GM-CSF (Novartis, Basel, Switzerland). Media, FCS, and sodium pyruvate were purchased from Gibco Life Technologies, Rockville, MD, USA.

Cytogenetic analysis

Chromosome banding analyses were performed on short-term cultures of bone marrow and peripheral blood samples. Methods of cell culture, chromosome preparation, and staining by a modified Giemsa-banding technique have been reported previously.[23]

Fluorescence in situ hybridization (FISH)

PAC clones representing the region surrounding the *EVI-1* gene in 3q26 were selected using the Genome Browser of the University of California at Santa Cruz (*http://genome.ucsc.edu*). All clones were from the RPCI-11 library, created by M. Takeno and K. Osoegawa,[24] and were purchased from the Resource Centre of the German Human Genome Project (RZPD) in Berlin. PAC clones 258G22, 73F18, 368I23, and 33A1, collectively called the centromeric PAC clones, together cover a region of approximately 1Mb that includes *EVI-1* and the region centromeric to it. PAC clones 816J6, 362K14, 379K17, 543D10, and 469J4, collectively called the telomeric PAC clones, represent a 1Mb region telomeric to *EVI-1*. The two groups of clones are separated by a gap of approximately 500kb (Figure 1A). Fluorescence *in situ* hybridization on metaphase cells from a healthy individual confirmed the localization of all PAC clones to 3q26 and the absence of additional hybridization signals, and demonstrated a signal to noise ratio appropriate for FISH experiments.

For interphase FISH, equal amounts of restriction digested PAC DNAs were mixed and labeled with Spectrum Orange (centromeric PAC clones) or Spectrum Green (telomeric PAC clones) by nick translation[25] (all labeling reagents from Vysis, Downers Grove, IL, USA). Then, 30 ng of each of the centromeric and the telomeric PAC clones, blocked with 1.2 μg human placenta DNA and 0.6 μg cot-1 DNA and dissolved in 3 μL Hybrisol VII (Oncor, Gaithersburg, MD, USA), were spotted on preparations of fixed, pepsin- and formaldehyde-treated, dehydrated cell nuclei and covered with a coverslip 10mm in diameter. DNA was denatured for 10 min at 72°C, and hybridization was carried out at 37°C overnight. After stringent washing, chromosomal DNA was counterstained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI). Results were analyzed on a Zeiss Axiophot microscope (Zeiss, Jena, Germany) equipped with a dual bandpass filter. At least 200 interphase cells were scored for each sample.

In order to account for the fact that the PAC clones labeled with the two fluorochromes are separated by a genomic region of 0.5Mb, red and green signals were scored as co-localizing if they were located up to one signal diameter apart, and as separated if their distance from each other was more than one signal diameter.[26] Threshold values for true positivity were calculated from the average percentages, plus three times the standard deviations, of nuclei falsely positive for each of the three aberrant hybridization patterns in 20 samples from patients without cytogenetically detectable 3q26 rearrangements.

Results

Selection and testing of PAC clones

Figure 1A illustrates the positions of the 3q26 PAC clones used for interphase FISH, and Figure 1B shows the expected and observed hybridization patterns on cell nuclei without and with 3q26 aberrations. Hybridization of these probes to cell nuclei without 3q26 aberrations yielded two pairs of co-localizing red and green signals. 3q26 rearrangements either separated one red/green signal pair, or, if breakpoints were located further centromeric or telomeric, split one of the red or the green signals, respectively. Threshold values for true versus false positivity were established using samples from 20 patients with myeloid malignancies without cytogenetically detectable 3q26 rearrangements, and are summarized in Table 1.

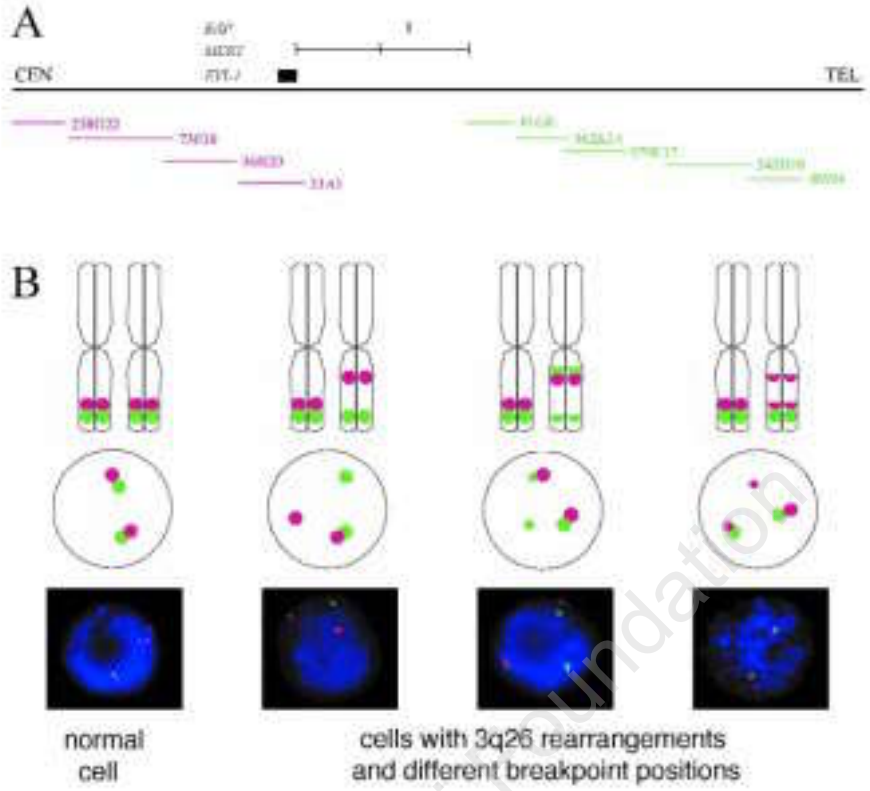

Figure 1. The 3q26 interphase FISH assay. A) Schematic representation of the 3q26 breakpoint region. cen, centromeric, tel, telomeric. The positions of the genes *EAP*, *MDS1*, and *EVI-1* are indicated; boxes represent exons and horizontal lines introns. PAC clones are from the RPCI-11 library, and are drawn in red or green, according to the fluorescent dyes used to label them for interphase FISH. Positional information for genes and PAC clones was derived from the Genome Browser of University of California at Santa Cruz (*http://genome.ucsc.edu*), Aug. 2001 freeze. B) Expected and observed signal patterns of the 3q26 FISH probe. In metaphase and interphase cells without 3q26 rearrangements, the red and green signals co-localize on the two chromosomes 3. In cells with 3q26 rearrangements, one red/green signal pair becomes separated, or, if the breakpoint is located further centromeric or telomeric, one of the red or one of the green signals is split, respectively. In the latter two cases, this leads to the observation of two red/green signal pairs and one additional red or green signal with diminished intensity.

The interphase FISH assay was further validated using cell lines with 3q26 rearrangements. The vast majority of HNT-34, JH, and Mutz-3 interphase nuclei exhibited signal patterns consistent with the presence of a 3q26 aberration. When the t(3;3) containing HNT-34 cells were mixed in varying proportions with MPD cells, which do not contain a 3q rearrangement, the percentage of cells with aberrant signal patterns correlated very well with the relative input of HNT-34 cells (*data not shown*).

Analysis of patients' samples

Bone marrow or peripheral blood samples from 17 patients with 3q26 rearrangements, as deter-

Table 1. Threshold values for the three types of aberrant hybridization patterns shown in Figure 1B.

	Average ± SD	*Threshold value*
separation of one red/green signal pair	2.4±2.6 %	10.2 %
splitting of one red signal	0.1±0.3 %	1 %
splitting of one green signal	0.1±0.4 %	1.3 %

The average occurrence and standard deviation (SD) of each pattern type was determined on samples from 20 patients with various types of myeloid malignancies without cytogenetically detectable 3q26 rearrangements. Threshold values were calculated from average values plus 3 standard deviations.

Table 2. Clinical characteristics and interphase FISH results of patients with 3q26 rearrangements.

Pat. no.	*Age/Sex*	*Diagnosis*	*BM/PB*	*chromosome 3*	*aberrant metaphase cells*	*% aberrant interphase cells*
1	51/M	M4	BM	t(3;21)	40/40	85.9 %
2	34/M	CML-AP	PB	t(3;21)	27/30	88.8 %
3	50/M	CML-BC	PB	t(3;21)	8/8	95.8 %
4	54/F	preB/M1	BM	t(3;3)	26/26	93.8 %
5	56/F	MDS	BM	t(3;3)	20/20	95.6 %
6	49/F	CML-BC	BM	t(3;3)	29/29	97.6 %
7	31/F	M0	PB	inv(3)	17/17	46.5 %
8	65/M	M4	BM	inv(3)	16/20	57.6 %
9	12/M	M2	BM	inv(3)	20/20	79.7 %
10	57/M	M1	BM	inv(3)	20/20	73.3 %
11	69/F	CML-AP	BM	inv(3)	20/20	56.4 %
12	73/F	AML	BM	inv(3)	16/16	75.7 %
13	49/M	M4	BM	inv(3)	20/20	71.9 %
14	47/F	M4	BM	inv(3)	20/20	40.9 %
15	75/F	M2	BM	inv(3)	20/20	43.1 %
16	42/F	M1	PB	inv(3)	12/12	79.0 %
17	37/F	M0	BM	inv(3)*	10/10	97.1 %

Pat. no., patient number. M, male, F, female. AML, acute myeloid leukemia; CML, chronic myeloid leukemia;MDS, myelodysplastic syndrome; M0-M4, FAB types of AML; preB/M1, biphenotypic acute leukemia; CML-AP, accelerated phase of CML, CML-BC, blast crisis of CML. BM, bone marrow; PB, peripheral blood. t(3;21) indicates the presence of a t(3;21)(q26;q22); t(3;3) indicates the presence of a t(3;3)(q21;q26);and inv(3) the presence of an inv(3)(q21q26), which was associated with an ins(6;3) in case 17 (marked by an asterisk).
The proportion of metaphase cells carrying the respective 3q rearrangement was determined by chromosome banding analysis. The percentage of aberrant interphase cells observed with the 3q26 FISH assay is indicated. In cases in which two different aberrant hybridization patterns were present and exceeded their respective threshold values, the sum of the percentages of aberrant interphase nuclei is given.

mined by chromosome banding analysis, were investigated using the interphase FISH assay. Clinical characteristics of the patients, as well as results of classical cytogenetic and interphase FISH analyses, are summarized in Table 2. In all cases, the expected aberrant hybridization patterns were detected in a large fraction of the interphase cells (Table 2, Figure 2). A representative subset of the samples with 3q26 rearrangements was re-evaluated, or rehybridized and re-evaluated, at a later time point, blind to the results of the first analysis. Results of these repeated analyses were comparable, with differences in the numbers of nuclei scored as aberrant being no larger than 11% (i.e., in the range of the threshold value for true positivity), but much lower in most cases.

Discussion

In this study, we established an interphase FISH assay for the detection of chromosome rearrangements affecting the *EVI-1* locus in band 3q26. The use of a probe representing, altogether, a genomic region of 2.5Mb allowed the efficient detection of inv(3), t(3;3) and t(3;21) rearrangements, despite the fact that the corresponding breakpoints are distributed over comparatively large genomic distances. The assay can be used to confirm or exclude the presence of 3q26 aberrations at diagnosis. Furthermore, during follow-up, it should provide a rapid and simple quantitative measure of the proportion of leukemic cells present in a sample. This assay complements a similar test we recently described for the 3q21 breakpoint region[27] in that

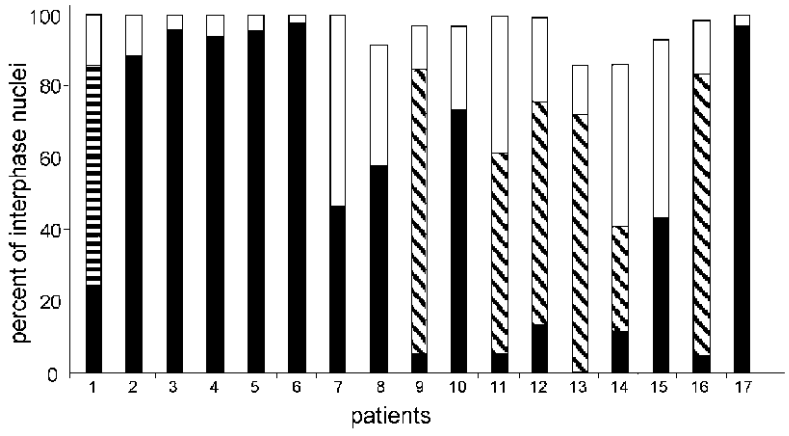

Figure 2. Analysis of samples with 3q26 rearrangements. Bone marrow or peripheral blood samples from 17 patients with t(3;21), t(3;3), or inv(3) were analyzed using interphase FISH. The patients' characteristics are summarized in Table 2. Black portions of bars, percentages of interphase cells exhibiting separation of one of the red/green signal pairs; diagonally striped portions of bars, percentages of interphase cells with splitting of one red signal; horizontally striped portions of bars, percentages of interphase cells with splitting of one green signal; white portions of bars, percentages of interphase cells with normal hybridization patterns. Total bar length may be less than 100%, because some interphase cells exhibited variable non-specific hybridization patterns that did not correspond to any of the patterns shown in Figure 1B.

it is able to detect not only the inv(3) and the t(3;3), but also the t(3;21) and, most likely, the t(3;12), both of which have 3q26, but not 3q21 breakpoints. Furthermore, the two assays can be used to independently confirm the involvement of the 3q21 and the 3q26 breakpoint regions in cases with inv(3) or t(3;3), which will be of particular interest in cases with insufficient numbers or low quality of metaphases, or with masked aberrations.[28]

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Pre-publication Report & Outcomes of Peer Review

Contributions

RW planned, designed, and supervised the study and interpreted the interphase FISH results. She drafted and wrote the manuscript. US carried out, analyzed, and interpreted the interphase FISH experiments. HR, HP-D, HG, IL, and CF analyzed and interpreted G-band patterns. They, as well as US, critically reviewed the manuscript and agreed to its final version. Primary responsibility for paper, all figures and tables: RW.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous paper.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Cristina Mecucci, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Mecucci and the Editors. Manuscript received July 16, 2002; accepted November 12, 2002. In the following paragraphs, professor Mecucci summarizes the peer-review process and its outcomes.

What is already known on this topic

Chromosome aberrations involving 3q26 are well documented changes in myeloid malignancies. Among the most frequent changes, the inv(3)(q21q26) and the t(3;3)(q21;q26) lead to overexpression of EVI1 gene at 3q26. New transcripts derive from translocations such as t(3;12) or t(3;21). A poor prognostic value is associated with these entities.

What this study adds

The work describes for the first time a new approach to detect 3q26 anomalies based on a double color FISH in interphase nuclei.

Caveats

An important application of the described technology is its use in diagnosis and monitoring of 3q26-associated disorders overcoming the limits of conventional cytogenetics. However, large clinical studies are needed to validate the technique by comparing this interphase FISH approach with karyotyping or other molecular investigations.