**High Frequency of Monoallelic Retinoblastoma Gene Deletion in B-Cell Chronic Lymphoid Leukemia Shown by Interphase Cytogenetics**

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Inactivation of the retinoblastoma tumor-suppressor gene (RB-1) has been associated with tumorigenicity in various human malignancies. In chronic lymphoid leukemias of B-cell origin (B-CLL) an involvement of RB-1 has been suggested based on cytogenetic data. We examined RB-1 and its chromosomal locus 13q14 in 35 cases of B-CLL by dual-color in situ hybridization to interphase nuclei and by G-banding analysis of metaphase chromosomes. In one patient the tumor cells showed a monosomy 13, and in three other cases deletions involving or encompassing band 13q14 were detected by conventional cytogenetic analysis. In contrast, in situ hybridization to interphase nuclei showed a monoallelic RB-1 deletion in 11 cases (31%). One pt showed a translocation with the breakpoint in 13q174 on G-banding, but on in situ hybridization analysis the RB-1 signals were not affected. Our data show that RB-1 deletions can be diagnosed accurately by in situ hybridization on the one-cell level. The frequency of RB-1 deletions detected in this study is significantly higher than previously assumed in B-CLL, and seems to be in the same range as in retinoblastoma.

**MATERIALS AND METHODS**

**Patients.** From October 1990 to May 1992, cytogenetic analysis using G-banding and CISH hybridization was performed on 32 pts with B-CLL and three pts with B-prolymphocytic leukemia (B-PLL). Pt samples were collected prospectively from our hematology outpatient clinic. Classification of the leukemias was performed according to the proposals established by the French-American-British (FAB) Cooperative Group. Immuno phenotypic data were available in 30 of the 32 pts with B-CLL: 29 pts were CD19+CD5-, and 1 pt was CD19+CD5+. One pt had Rai stage I, 11 Rai stage II, 12 Rai stage III, and 8 Rai stage IV disease. The three pts with B-PLL had more than 55% prolymphocytes in the blood and strong slg expression; all three had advanced disease and were entered on a treatment protocol of the European Organization for Research and Treatment of Cancer (EORTC) using deoxycoformycin (Pentostatin). Twenty-six pts had chemotherapy before cytogenetic analysis. None of the pts had a history of RB.

**G-banding analysis.** Mononuclear cells were isolated from blood, lymph node, or splenic tissue over a Ficoll gradient and cultured at

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a concentration of $1 \times 10^6$ cells/mL in the presence of anti-human IgM (10 ng/mL) (Jackson Research Immunobio), West Grove, PA), B-cell growth factor (10%) (Cellular Products Inc, Buffalo, NY), and phorbol 12-myristate 13-acetate (1 to 10 ng/mL) (Sigma, St Louis, MO) or calcium-ionophore (1 pmol/L) (Calbiochem, La Jolla, CA). Cells were harvested from 3- to 5-day cultures. After mitotic arrest (colcemid 0.05 to 0.07 µg/mL, 20 to 90 minutes; ethidium bromide 10 µg/mL, 90 minutes) and hypotonic treatment (0.075 mol/L KCl, 15 minutes, 37°C), cells were fixed in methanol/acetic acid (3:1). Part of the chromosome preparations were G-banded with Wright's stain for karyotype analysis. Metaphases were obtained in 31 pts (range: 10 to 28, mean 18.4; see also Table 2). Karyotypes were described according to the International System for Cytogenetic Nomenclature.25

Blood smear. In one pt blood smears previously stained by Wright's were used for in situ hybridization. After destaining and fixation by incubation in methanol for 3 x 10 minutes at room temperature and washing in phosphate-buffered saline (PBS), treatment with pepsin was performed as described below to improve probe penetration.

In situ hybridization. Sixteen λ-phase clones spanning the whole 200 kb of RB-1 were generously provided by Dr Thaddeus P. Dryja (Harvard Medical School, Cambridge, MA).29 Cosmid clone c518 (D21S55) mapping to 21q22.3 served as a positive control for hybridization efficiency and was kindly provided by Paul Watkins (Gaithersburg, MD) and Dr Katherine Klinger (Integrated Genetics, Framingham, MA). Labeling of the DNA probes and CISH hybridization were performed as previously described.28 Briefly, the hybridization mixture contained 16 µg/mL biotinylated RB-1 DNA (pool of 16 λ-phase clones), 8 µg/mL digoxigenin-labeled c518-DNA, 300 µg/mL human Cot-1 DNA (Bethesda Research Laboratories, Gaithersburg, MD), and 700 µg/mL salmon-sperm DNA. After hybridization overnight and posthybridization washes the DNA probes were detected using avidin-conjugated fluorescein isothiocyanate (FITC) (Vector, Burlingame, CA) and anti-digoxigenin-conjugated rhodamine (Boehringer, Mannheim, Germany), respectively. After counterstaining with 4,6 diamidino-2phenylindole dihydrochloride (DAPI) the slides were evaluated on a conventional fluorescent microscope (Zeiss, Oberkochen, Germany). RB-1 hybridization signals were enumerated in 120 to 150 interphase nuclei for each specimen. To prevent false-positive results caused by inadequate hybridization or chromatim loss, analysis was only performed on slides with high hybridization efficiency, indicated by two c518 hybridization signals in more than 90% of the nuclei. If hybridization efficiency was inadequate, cells were pretreated with protease-K (2 µg/mL) or pepsin (0.1 mg/mL) for 10 minutes at 37°C and postfixed in 1% to 4% paraformaldehyde (Sigma) for 10 minutes to improve probe penetration.

Illustrations were produced using a cooled charged coupled device (CCD) camera (Photometrics, Tucson, AZ). Separate images were obtained for each fluorochrome and overlayed after digital processing as described.37

RESULTS

G-banding analysis. Evaluable metaphases were obtained in 31 of 35 pts. Sixteen of 31 pts (52%) had chromosomal abnormalities. Three pts had trisomy 12 and two pts 14q32 abnormalities (Table 2: pt 5 and pt 11). Aberrations of chromosome 13 were found in five pts (Table 2: pt 1 through pt 5): pt 2 showed a translocation t(1;13) with a breakpoint within band 13q14 (Fig 1A); pts 1, 3, and 4 had interstitial deletions (see Fig 1B) of which two had deletion breakpoints within 13q14 (pt 3 and pt 4), whereas the deletion encompassed 13q14 in pt 1; pt 5 had a monosomy 13.

Delineation of RB-1 by in situ hybridization. The RB-1 probe showed bright hybridization signals on metaphase spreads from lymphocyte preparations of five healthy probands (Fig 1C) and more than 86% of interphase nuclei showed two hybridization signals (for details see Table 1 and Fig 2). On average, 6.0% (mean; range: 1% to 9%; SD: 2.0%) of the control nuclei had only one signal. In some of these nuclei, this finding appeared to be caused by close juxtaposition of two signals, whereas in others the cause could not be identified. By analogy with a previous study,28 a deletion was diagnosed if pts exhibited highly significant percentages of cells with a single RB-1 signal, ie, specimens showing more than 12% (mean + 3 SD) of nuclei with only one signal (cut-off level in Fig 2).

RB-1 deletions in patient material. The frequency of cells exhibiting one RB-1 signal is shown for all probands and pts in Fig 2. Eleven of 35 pts (31%) had a highly significant number of interphase nuclei with a single RB-1 signal. Clinical and cytogenetic data of these 11 pts are presented in Table 2: The frequency of cells with a single hybridization signal ranged from 31% to 85%, with a mean of 67%. To prove that these numbers were not caused by inefficient hybridization, cohybridization was performed using a differentially labeled chromosome 21 cosmids probe (c518)39 that results in hybridization signal intensities comparable with those generated by the RB-1 probe set. Following dual color detection, the distribution of c518 signals was evaluated in all cells exhibiting only one RB-1 signal in probands and pts (Tables 1 and 2). In pts where a Rb gene deletion was diagnosed, the number of cells showing one RB-1 signal and two c518 signals was almost as high as the number of cells with one RB-1 signal counted by single-color evaluation (compare last two columns of Table 2). Therefore, the high number of cells exhibiting one RB-1 signal cannot be attributed to technical limitations but truly represents a monoallelic Rb gene deletion. All of the specimens with RB-1 deletion also showed a varying percentage of cells exhibiting two RB-1 hybridization signals (range: 5% to 67%), indicating the presence of normal cell populations (Fig 1D). As shown by the internal control, the percentage of normal and chromosomally altered cells, respectively, was assessed with high accuracy.

In pt 8 the deletion of RB-1 was confirmed by CISS hybridization of a blood smear. A high number of cells morphologically appearing as lymphocytes exhibited one hybridization signal (83%, 110 of 133 cells), whereas 86% (19 of 22 cells) of the granulocytes had two signals (Fig 1E). This finding indicated that the deletion of RB-1 in this pt originated from a somatic mutation event in a lymphoid cell lineage.

Of the 11 pts showing a RB-1 deletion in interphase analysis, 2 pts had high numbers of mitotic cells: in pt 9 all 15 and in pt 8 29 of 30 metaphases had two RB-1 signals, whereas interphase analysis showed RB-1 deletions in 51% and 85% of the nuclei, respectively (Fig 1F). This observation indicated a low in vitro mitotic activity of the cells carrying the deletion. In both pts a normal karyotype was diagnosed by G-banding (Table 2).

Blood and lymph node specimens were available in three pts. On in situ analysis, in one of these (pt 1) the lymph node specimen exhibited 32% of cells with RB-1 deletion whereas...
the blood specimen was normal. This observation was confirmed by karyotype analysis which showed a del(13)(q12q22) in the lymph node but not in the blood specimen (Table 2).

Our study showed chromosomal abnormalities involving band 13q14 or deletions of the Rb gene in 12 of 35 pts with B-CLL. CISS alone detected RB-1 deletions in 11 pts. Only 5 of these 11 pts exhibited abnormalities of chromosome 13 on conventional cytogenetic analysis. In one pt (pt 2), G-banding showed a translocation involving band 13q14 that did not affect the RB-1 hybridization signal, i.e., the translocation did not result in the loss of a RB-1 allele; whether the translocation breakpoint involves the Rb gene resulting in inactivation is unknown.

**DISCUSSION**

We performed a combined metaphase and interphase cytogenetic study by means of conventional G-banding and CISS hybridization with the intention to analyze the chromosomal region 13q14 and, more specifically, the Rb gene in 32 cases of B-CLL and three cases of B-PLL.

Our data indicate that a chromosomal deletion of RB-1 is more common in chronic lymphoid neoplasms than previously thought. A review of published chromosome data evaluated at 17 different centers showed abnormalities of 13q in 13% of cases whereas our data showed 31% of pts with RB-1 deletion. This substantial increase in incidence is unlikely to result from an unusual patient population, because the frequency of trisomy 12, and 13q alterations, as detected by G-banding analysis, is consistent with previous reports. Six of the 11 cases with RB-1 deletion showed no abnormality of chromosome 13 on G-banding analysis and could only be diagnosed by in situ hybridization. Therefore, the high number of RB-1 deletions detected in this study is based on the improvement in diagnostic accuracy by the additional interphase cytogenetic approach, which helps to avoid several pitfalls of conventional chromosome analysis.

First, using suitable probe sets, in situ hybridization gives informative results in almost all nuclei of a specimen. Instead of a limited number of metaphases, a large number of cells can be evaluated rapidly, and the result becomes more representative. Furthermore, in situ hybridization allows analysis of dividing as well as nondividing cell populations, and it does not rely on a successful mitogen stimulation to gain an adequate number of metaphases. These advantages are particularly important in diseases characterized by low in vitro mitotic activity, such as B-CLL. As exemplified by pt 8 and pt 9, no clonal chromosomal aberration and no RB-1 deletion were detected on metaphase spreads, whereas interphase nuclei showed an RB-1 deletion. From our study it cannot be concluded whether the metaphases analyzed originated from normal B or T lymphocytes or from leukemic cells. However, a previous study indicated that mitotic cells with a normal karyotype present in specimens with clonal chromosomal abnormalities often represent nonleukemic T lymphocytes.

The technical limitations of an approach solely based on metaphase analysis might also contribute to contradictory results regarding the prognostic significance of cytogenetic aberrations in different studies. Thus, it could be expected that the improvements in the diagnostic accuracy of chromosomal aberrations by interphase analysis will provide the basis for more consistent correlations between chromosomal changes and prognosis.
In addition to the superior sensitivity, multicolor in situ hybridization also increases the specificity in the detection of chromosomal aberrations. In previous cytogenetic studies on B-CLL, 13q aberrations were among the most common chromosomal abnormalities, and an involvement of the Rb gene was suspected. On the other hand, Southern blot analysis, until recently, did not provide evidence for a frequent involvement of RB-1 in B-CLL. With the use of in situ hybridization we were able to show RB-1 deletions in an unexpectedly high number of cases. Therefore, the possible pathogenetic role of the Rb gene in the initiation or progression of B-CLL is reinforced by this study. It is intriguing that the incidence of RB-1 deletions detected is comparable with that described in Rb. Our finding adds to the growing data supporting the idea of a more general role of RB-1 in human tumorigenesis.

Most recently, another group reported RB-1 deletions in pts with B-CLL in a frequency comparable with our results by applying quantitative Southern blot analysis following T-cell depletion. By comparison with previous conventional cytogenetic studies, this method is apparently more sensitive in detecting RB-1 deletions in B-CLL. However, no conclusion about the RB-1 status on the single-cell level can be drawn. Therefore, in addition to the well-known difficulties in the interpretation of band densities, the diagnostic usefulness of Southern blot analysis might be especially limited in pts who have a loss of RB-1 in a minority of cells (ie, pt 1 and pt 4 in Table 2). In contrast, the demonstration of RB-1 deletions on the cellular level by CISS hybridization is a more direct and thereby possibly more informative form of analysis. The exact percentage of cells carrying the deletion can be determined and correlated with stage, response to therapy, and survival, or combined with gene therapy approaches in the future.

The usefulness of in situ hybridization for the detection of numerical chromosome aberrations in B-CLL was previously shown using centromere-specific repetitive DNA probes. In this study we applied CISS hybridization to assess a structural aberration using a DNA probe set specifically delineating a gene of possible pathogenetic significance in B-CLL. Because a 200-kb DNA probe set spanning the entire Rb locus was used for in situ analysis, RB-1 deletion was detected only in nuclei that had lost one entire allele. The number of pts affected by RB-1 mutation is probably...
still underestimated in this study, because small structural aberrations or point mutations are not detectable with our method. Molecular analyses of Rb showed that in up to two thirds of the cases, other chromosomal mechanisms, rather than deletions, are responsible for ablation of Rb function.\(^{20,21}\) Therefore, further studies closing the gap to the molecular level of analysis are needed to completely assess the involvement of Rb-1 in chronic lymphoid leukemia. We have initiated a study to analyze Rb-1 by DNA sequencing to detect possible mutations in cases where no deletion was found, and to obtain detailed information about the status of the remaining Rb-1 allele in pts carrying a deletion.

Obtaining cytogenetic data beyond the level of metaphase cells provides high accuracy and more detailed information that might lead to a better understanding of the pathogenetic and prognostic significance of genetic findings in hematologic malignancies.

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