Detection of t(11;14) Using Interphase Molecular Cytogenetics in Mantle Cell Lymphoma and Atypical Chronic Lymphocytic Leukemia

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The chromosomal translocation t(11;14)(q13;q32) fuses the *IGH* and *CCND1* genes and leads to cyclin D1 overexpression. This genetic abnormality is the hallmark of mantle cell lymphoma (MCL), but is also found in some cases of atypical chronic lymphocytic leukemia (CLL), characterized by a poor outcome. For an unequivocal assessment of this specific chromosomal rearrangement on interphase cells, we developed a set of probes for fluorescence in situ hybridization (FISH). Northern blotting was performed for analysis of the cyclin D1 expression in 18 patients. Thirty-eight patients, with either a typical MCL leukemic phase (17 patients) or atypical CLL with an MCL-type immunophenotype, i.e., $CD19^+$, $CD5^+$, $CD23^{-/low}$, CD79b/sIgM(D)$^{++}$, and $FMC7^+$ (21 patients), were analyzed by dual-color interphase FISH. We selected an *IGH*-specific BAC probe (covering the *JH* and first constant regions) and a commercially available *CCND1* probe. An *IGH–CCND1* fusion was detected in 28 of the 38 patients (17 typical MCL and 11 cases with CLL). Cyclin D1 was not overexpressed in two patients with typical MCL and an *IGH–CCND1* fusion. In view of the poor prognosis associated with MCL and t(11;14)-positive CLL, we conclude that this set of probes is a valuable and reliable tool for a rapid diagnosis of these entities. *Genes Chromosomes Cancer 23:175–182, 1998.*

INTRODUCTION

Mantle cell lymphoma (MCL) is a non-Hodgkin's lymphoma (NHL) that was recently recognized as a specific entity in the revised NHL classification (Harris et al., 1994). The accuracy of this individualization is confirmed by clinical studies, all of which demonstrate the poor outcome of MCL patients (Coiffier et al., 1995; Fisher et al., 1995; Stewart et al., 1995; Teodorovic et al., 1995; Zucca et al., 1995). The normal counterpart of these malignant cells is well characterized (naive B-cells that home to and reside in primary lymphoid follicles and the mantle zones of secondary follicles). However, identification of malignant lymphocytes such as MCL cells may be a difficult challenge for pathologists. Although different immunologic, cytogenetic, and molecular characteristics may be useful for recognizing these malignant lymphocytes, none of them is constant and specific. Leukemic phases are frequent and may be inaugural in MCL. It may be difficult to distinguish the morphology of circulating abnormal cells in MCL from those in atypical chronic lymphocytic leukemia (CLL). Flow studies cannot always differentiate between these cell types. Like CLL cells, MCL cells coexpress the T-cell antigen CD5, panB antigens (CD19, CD20, and CD22), and surface IgM (and usually IgD). In contrast, FMC7 positivity, strong expression of B-cell receptor CD79b/sIg molecules, and weak expression of CD23 (or none) are specific for MCL cells (Lardelli et al., 1990; Matutes et al., 1994; Segal et al., 1995; Garand and Robillard, 1996; Weisenburger and Armitage, 1996). However, this typical MCL immunophenotypic profile may be encountered in some cases of typical CLL (Garand and Robillard, 1996).

Cytogenetic analyses in MCL usually show a t(11;14)(q13;q32), but approximately 40% of analyzed cases apparently lack this typical translocation (Leroux et al., 1991; Vandenberghe et al., 1992). On chromosome 14, breakpoints occur within the Ig heavy chain (*IGH*) gene, usually in the *JH*

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region. On chromosome 11, breakpoints are not tightly clustered, but occur over at least 130 kb upstream of the *CCND1* gene, which encodes the cyclin D1 protein (de Boer et al., 1993, 1995a, 1995b; Rimokh et al., 1993). Because of the dispersed 11q13 breakpoints, molecular studies (Southern blot or PCR experiments) fail to detect the rearrangements in 30–50% of cases (de Boer et al., 1993; Rimokh et al., 1994; Luthra et al., 1995). Theoretically, the best diagnostic test is the demonstration of cyclin D1 overexpression by use of Northern blotting (de Boer et al., 1993; Rimokh et al., 1993). However, this test is time-consuming and usually is not performed in routine analysis. Recently, a new competitive RT-PCR-based approach has been reported (Uchimaru et al., 1997). Despite encouraging results, this technique needs to be evaluated on a larger series of patients, which, unfortunately, is usually not available in routine laboratories.

In order to circumvent the lack of sensitivity of molecular techniques, we have developed a fluorescence in situ hybridization (FISH) approach to detect the *IGH–CCND1* rearrangements on interphase nuclei. After selection of adequate 14q32 and 11q13 probes, we have analyzed 38 patients with either a leukemic phase of MCL or an atypical CLL with an MCL-type immunophenotype.

MATERIALS AND METHODS

Patients

Seventeen patients with a histologic diagnosis of MCL according to the REAL classification (Harris et al., 1994) and a leukemic phase were selected for this study. We also analyzed 21 patients cytologically diagnosed as atypical CLL (20 cases with mixed-cell-type CLL and one patient with B-prolymphocytic leukemia) (Bennett et al., 1989), but with an immunophenotype characteristic of MCL (i.e., $CD19^+$, $CD5^+$, $CD23^{-/low}$, CD79b/$sIgM(D)^{++}$, $FMC7^+$) (Matutes et al., 1994; Garand and Robillard, 1996). Their clinical, morphologic, immunologic, and molecular characteristics are summarized in Tables 1 and 2. For 34 of these patients, analyses were performed on frozen bone marrow (2 cases) or peripheral blood (32 cases) samples. In two cases, we analyzed PB cytospins. In two other patients, we used BM cytogenetic preparations.

TABLE 1. Main Clinical, Histocytologic, Molecular, and Immunologic Characteristics

Patients	Sex	Age (year)	Clinical involvement[a]	Cytology[b]	Histology[c]
1	F	57	ADP, SMG	PL	MCL
2	M	42	ADP, SMG, DT	PL	MCL
3	F	74	ADP, SMG	PI	MCL
4	M	59	SMG	PL + PLY	MCL "BL"
5	M	66	ADP, SMG	PL	MCL
6	M	87	ADP	PL + PLY	MCL
7	F	41	ADP, SMG	PL + PLY	MOL
8	F	73	ADP, SMG, liver	"Blastoid"	ND
9	F	73	ADP, SMG	PL	MCL
10	F	43	ADP, SMG, liver, thyroid	PL	MCL
11	M	57	ADP, SMG	PL + PLY	MCL "BL"
12	M	71	ADP, SMG	PL + PLY	MCL
13	M	57	ADP, SMG	PL	MCL
14	M	51	ADP, SMG, skin	PL	LPL
15	F	48	ADP, SMG, liver	SL	MCL
16	F	66	ADP, skin	PL + PLY	MCL
17	F	71	ADP, SMG, liver	"Blastoid"	MCL "BL"
18	F	85	None	PL	ND
19	M	69	None	PL + PLY	ND
20	M	69	SMG, liver	PL + PLY	ND
21	M	72	SMG	PL + PLY	LPL
22	F	94	SMG, DT, CNS	PL	ND
23	M	67	ADP, SMG, liver	PL	LPL
24	F	75	SMG	PL + PLY	SLL
25	M	73	SMG	PL	ND
26	F	82	None	PL	SLL
27	M	55	ADP, SMG	PLY	PLL
28	M	51	SMG	PL + PLY	ND
29	M	66	SMG	PL + PLY	ND
30	M	72	ADP, SMG	PL	ND
31	M	77	SMG	PL	ND
32	F	67	None	PL	ND
33	F	56	ADP, SMG	PL	MCL
34	M	74	SMG, liver	SL	ND
35	M	69	ADP, SMG	PL	MCL "BL"
36	M	83	ADP, SMG	PL	ND
37	F	84	None	PL	ND
38	F	54	None	PL	ND

[a]ADP = adenopathy; SMG = splenomegaly; DT = digestive tract; CNS = central nervous system.
[b]PL = pleiomorphic lymphocytes; PLY = prolymphocytes; SL = small monomorphic lymphocytes.
[c]ND = not done; LPL = lymphoplasmacytic lymphoma; PLL = prolymphocytic leukemia; SLL = small lymphocytic lymphoma; MCL = mantle cell lymphoma; "BL" = blastoid variant.

Immunologic Analyses

Peripheral blood (n = 35) or bone marrow (n = 3) mononuclear cells were isolated using Ficoll-Hypaque and analyzed by dual-color fluorescence flow cytometry (FACSCALIBUR, Beckton-Dickinson). Different combinations of monoclonal antibodies—directly labeled with fluorescein isothiocya-

TABLE 2. Immunologic and FISH Characteristics

Patients	Phenotype[a]	% B-cells[b]	% CD5+ B-cells	Cytogenetics (C), Southern (S), and Northern (N)[c]	FISH[d] (configuration)	% Cells with fusion
1	A (IgD−)	64	48	ND	R (A)	62
2	C	73	73	C−, N+, S+	R (A)	65
3	C	92	77	ND	R (B)	94
4	C	87	85	N−	R (A)	85
5	A (CD5−)	97	0	S+	R (A)	93
6	C	74	74	ND	R (A)	80
7	C	90	90	C+, N+, S−	R (B)	90
8	A (IgD−)	97	91	C+, S−	R (A)	83
9	C	88	88	ND	R (A)	79
10	C	75	75	N+	R (A)	68
11	C	61	57	N+	R (B)	60
12	C	85	85	N−	R (A)	82
13	C	78	76	ND	R (A)	70
14	C	55	43	ND	R (A)	51
15	C	65	61	N+	R (A)	60
16	C	79	79	ND	R (A)	75
17	C	79	78	N+, S+	R (A)	80
18	C	78	75	N+	R (A)	73
19	C	50	48	N+	R (A)	45
20	C	89	89	N+	R (A)	91
21	C	77	69	N−, S−	NR	2
22	C	73	72	N+	R (A)	70
23	C	64	55	N−	NR	1
24	C	86	31	ND	NR	3
25	A (IgD−)	88	88	ND	NR	2
26	C	93	82	N−	NR	2
27	A (IgD−)	88	88	N−	NR	3
28	C	73	50	N−	NR	1
29	C	86	84	N+	R (A)	81
30	C	83	78	ND	R (A)	92
31	C	55	55	ND	R (A)	54
32	C	82	31	ND	NR	1
33	C	71	70	ND	R (A)	63
34	A (IgD−)	79	71	ND	R (A)	84
35	C	73	70	ND	R (A)	68
36	A (IgD−)	66	66	ND	R (A)	60
37	C	41	41	ND	NR	2
38	C	84	84	ND	NR	2

[a]C = common ($CD19^+$, $CD5^+$, $CD23^{-/low}$, $FMC7^+$, $CD79b^{++}$, $sIgMD^{++}$); A = atypical (missing antigen).
[b]Cells with a B-phenotype: $CD19^+/CD22^+/sIg^+$.
[c]C+ = presence of a t(11;14) in the karyotype; N+ = cyclin D1 overexpression; S+ = Southern blot rearrangement at MTC locus.
[d]R = rearrangement (fusion 11q13–14q32); NR, no rearrangement.
Configuration A corresponds to a split of the *CCND1* probe; B, to the absence of a splice.

nate (FITC) or phycoerythrin (PE)—were used: CD5/IOT1a-FITC and CD19/IOB4-PE (Immunotech); CD23/B6-FITC (Coultronics) and CD22/leu14-PE (Beckton-Dickinson); FMC7-FITC (Seralab) and CD79b-PE (Immunotech). Surface Ig expression (μ, δ, γ, κ, and λ chains) was analyzed with FITC-goat F(ab')2 polyclonal antibodies (Kallstad).

Probes

The 11q13 probe was purchased from Vysis. This probe covers 300 kb, is centered on the *CCND1* locus, and contains both *MTC* and *FGF4* loci, but not the *FGF3* locus (Fig. 1). The 14q32 probe was selected from a BAC library, using a J1–J6 cDNA probe. Mapping of this probe was performed using PCR with primers specific for the switch regions Sμ, Sγ, Sα, and Sϵ, according to Bergsagel et al. (1996). The 11q13 probe was directly labeled with SpectrumOrange. The 14q32 probe was labeled with biotin in a regular random priming reaction. In order to test the accuracy of this set of probes, we first tested them on two samples with a cytogenetically proved t(11;14) (patients 7 and 8) (Fig. 2).

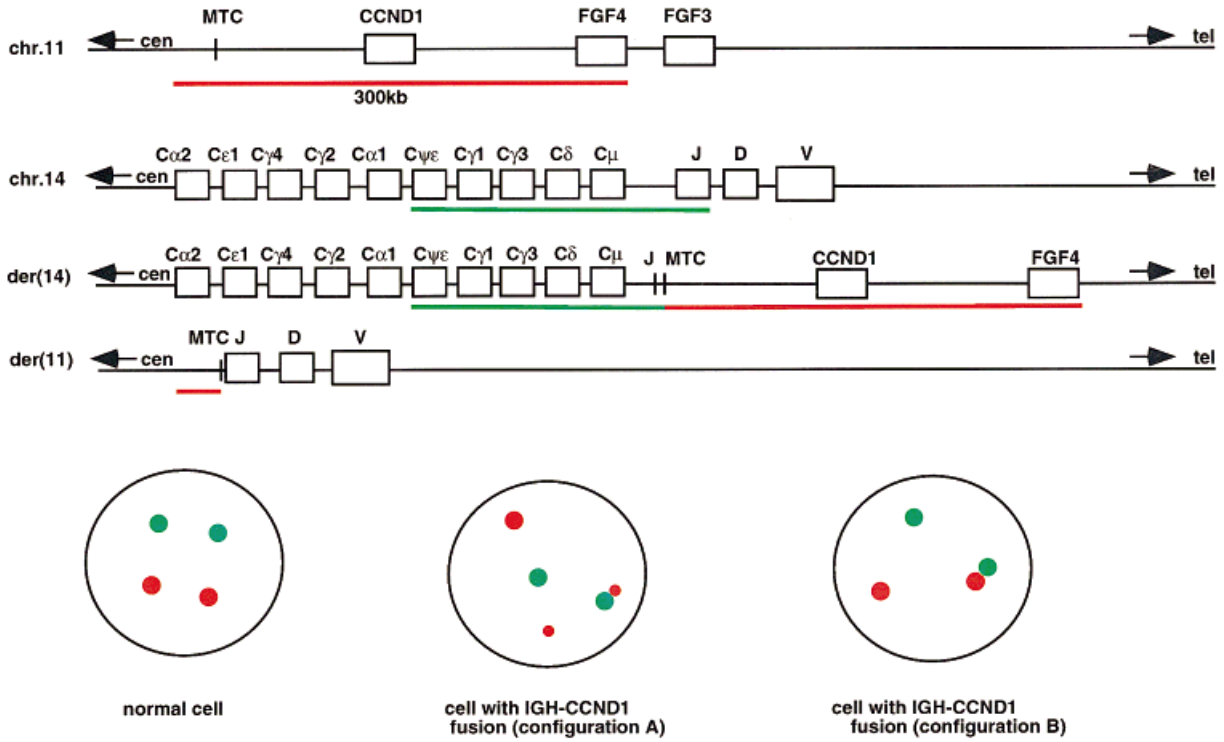

Figure 1. Schematic representation of the probe mapping and configuration on interphase nuclei. The lines and dots represent the 11q13 (red) and the 14q32 (green) probes.

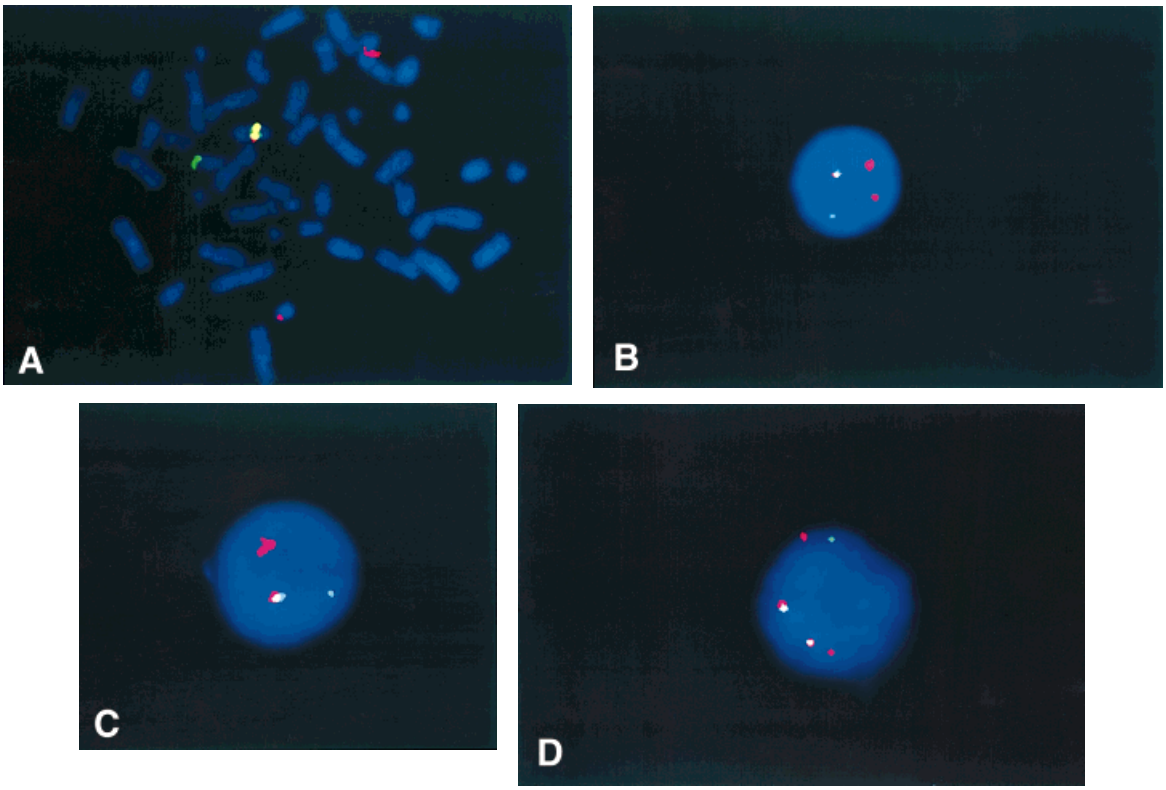

Figure 2. Green signals correspond to the 14q32 probe, red signals to the 11q13 probe, and yellow signals to the colocalization of the two probes. **A:** Hybridization on an MCL patient metaphase showing the 11q13 probe splitting and the colocalization of both probes on the der(14). **B:** A typical cell with a type A *IGH–CCND1* fusion. **C:** A typical cell with a type B *IGH–CCND1* fusion. **D:** An atypical configuration with a probable duplication of the der(14) (patient 30).

FISH

Thawed BM or PB cells were washed in PBS and separated into two samples, one for immunologic analysis and one for FISH analysis. The latter sample was fixed three times in methanol/acetic acid (3/1) and then dropped on slides. Slides were incubated for 30 min in 2 × SSC at 37°C and then dehydrated. They were then denatured in 70% formamide at 73°C for 90 sec and dehydrated in a cold alcohol series. Two hundred nanograms of the 14q32 probe was mixed with 1 μg of Cot-1 DNA (Gibco, Gaithersburg, MD), denatured for 5 min, and preannealed at 37°C for 15–30 min. The 11q13 probe was denatured for 5 min and then mixed with the 14q32 probe. The probes were then applied to slides, covered with a coverslip, and sealed with rubber cement. After overnight hybridization, slides were washed in 2 × SSC at 73°C for 5 min and then rinsed in 2 × SSC/0.1% Triton-X100. Detection was performed using FITC-avidin for 10 min at 37°C. After a final wash in 2 × SSC/0.1% Triton-X100, nuclei were counterstained with DAPI.

One hundred to 200 nuclei were then scored using an epifluorescence microscope (Leica) equipped with a triple-band-pass filter. In order to define the sensitivity of this set of probes, we tested them on 10 control patients. For controls, we chose five patients with non-B-cell hematologic malignancies (3 acute myeloid leukemia patients and 2 chronic myelocytic leukemia patients in chronic phase) to avoid any *IGH* rearrangement, and five normal volunteers. We used bone marrow samples from the five patients and peripheral mononuclear cells from the volunteers. Slide preparation and FISH experiments were performed under the same conditions as for MCL samples.

Molecular Analyses

For 18 patients, RNA was extracted from PB according to Chomczynski and Sacchi (1987). Northern blot analyses were performed with a cDNA cyclin D1 probe, enabling the detection of the two main transcript species of 4.5 and 1.2 kb. For each patient, the RNA amount and quality were controlled by use of a GAPDH-specific probe. We also performed Southern-blot analyses by using an *MTC*-specific probe in six patients.

RESULTS

Morphologic, Immunophenotypic, and Histopathologic Features

According to the morphology and immunophenotype of circulating abnormal cells and the histopathologic findings, patients were classified in two groups: MCL in leukemic phase and atypical CLL with an MCL immunophenotypic profile. Seventeen cases fulfilled the histopathologic criteria for MCL (Harris et al., 1994), including four "blastoid" variants. In this group, circulating abnormal cells (mean = 9×10^3/μl; range, 3–600 × 10^3/μl) were pleiomorphic lymphocytes with irregular, cleaved, nucleolized nuclei in 14 patients. In addition, blastoid cells were present in two cases histologically classified as blastoid MCL variant. One patient had small monomorphic lymphocytes, which were morphologically indistinguishable from common CLL. Flow studies revealed a typical MCL profile in 15 cases. IgD expression was absent in one case, and CD5 was negative in another.

In the second group, the initial absolute lymphocyte count was increased in all 21 patients (mean = 21×10^3/μl; range, 7–133 × 10^3/μl), morphologically classified as mixed cell type CLL in 20 cases and prolymphocytic leukemia (PLL) in one. Histopathologic data were available for six of these cases: three lymphoplasmocytoid lymphomas, two small lymphocytic lymphomas, and one PLL. Circulating abnormal lymphoid cells showed a typical MCL immunophenotypic profile in 17 cases and absence of IgD expression in four.

Validation of Probes

Fluorescence in situ hybridization analysis showed bright green and red signals in each experiment (Fig. 2). Analysis of the two samples with t(11;14) revealed one green signal on the normal chromosome 14, one red signal on each chromosome 11 [the normal one and the der(11)], and one yellow signal on the der(14). On controls, in the absence of *IGH–CCND1* rearrangement, two green signals (corresponding to the 14q32 probe) and two red signals (corresponding to the 11q13 probe) were detected. Only cells with yellow signals or colocalized signals (without any interval between the two signals) were considered positive for *IGH–CCND1* fusion. In controls, we analyzed 200 to 300 nuclei/control (2,700 nuclei total) and found colocalized signals in 1.8–3.7% of the cells (mean false positivity = 2.9%). Using this set of probes, the cutoff for positivity was fixed at 5.9% (mean + 3 S.D.).

In *IGH–CCND1* rearrangements, two different configurations were found (Fig. 1). The first configuration (type A) was one green signal (normal chromosome 14), two red signals [one corresponding to the normal chromosome 11 and one corresponding to the der(11)], and one yellow signal [corresponding to the colocalization of the two probes on the der(14)]. An atypical form of this configuration was found in one patient with two yellow signals instead

of one (patient 30). The second configuration (type B) was one normal green signal (normal chromosome 14), one red signal (normal chromosome 11), and one yellow signal [der(14)]. The type A configuration corresponds to a breakpoint on chromosome 11 between the *MTC* and *FGF4* loci, therefore splitting the probe. The patient with a variant form probably shared a duplication of the der(14) chromosome. The type B configuration corresponds to a break centromeric to the *MTC* locus, upstream from the 11q13 probe.

t(11;14) in Patients

We found an *IGH–CCND1* rearrangement in 28 of the 38 patients (Table 2). Among the 28 patients with colocalized signals, 25 displayed the type A configuration and three displayed type B. Comparison of percentages of clonal cells as determined by immunophenotype and percentages of cells with fusion showed an excellent correlation (Table 2). The *IGH–CCND1* fusion was found by FISH in all patients with MCL in leukemic phase, whereas only half of atypical CLL patients (11/21) displayed the rearrangement. In this latter group, a t(11;14) was found on both conventional cytogenetic and FISH analyses in one patient histologically classified as lymphoplasmacytic lymphoma.

Molecular Analyses

PCR experiments with the BAC probe showed the presence of specific PCR products with the Sμ, Sγ, and Sε primers, whereas no amplification was obtained with the Sα primers. We concluded that the probe maps within the JH, Cμ, $C\gamma_4$, $C\gamma_2$, and $C\epsilon_2$ regions (Fig. 1). Among the 18 patients with analyzable RNA, 11 had cyclin D1 overexpression, all of them displaying 11q13/14q32 fusion. Cyclin D1 was not detectable in two patients with typical MCL in leukemic phase, despite an *IGH–CCND1* fusion. In contrast, the five patients with atypical CLL and a t(11;14) using FISH overexpressed cyclin D1. Southern blot experiments using an *MTC*-specific probe showed a rearrangement in three cases, all of them displaying an *IGH–CCND1* fusion. Among the three patients without any rearrangement with this probe, two did show an *IGH–CCND1* fusion (one patient with each configuration) and one did not show any fusion.

DISCUSSION

Patients with MCL have in common advanced age, a marked male predominance, generalized lymphadenopathy (including splenomegaly), frequent BM involvement, and a poor outcome with standard therapies (Garcia-Conde and Cabanillas, 1995; Pittaluga et al., 1995; Weisenburger and Armitage, 1996). Even if typical cases are easily recognized from morphologic and immunologic analyses, this may be a difficult challenge for pathologists in atypical cases or when no biopsy specimen is available.

So far, four studies in which FISH was used to detect t(11;14) have been reported. The first used a dual-color FISH approach with 14q32- and 11q13-specific cosmid probes (Monteil et al., 1996). However, this study had a poor sensitivity (23%). The second study also used a dual-color technique, but with 11q13 probes only (Coignet et al., 1996). Four MCL samples were analyzed and were found to be rearranged with this set of probes. The third published study used a different FISH technique, namely, fiber-FISH using 11q13- and 14q32-specific probes (Vaandrager et al., 1996). These investigators found an 11q13–14q32 rearrangement in 19 of 20 MCL. However, this technique is not used routinely. Finally, a recent study using FISH with an 11q13 YAC probe on atypical CLL cases showed that a t(11;14) was present in 25% of patients (Cuneo et al., 1997). However, the use of single-color FISH with an 11q13 probe does not allow the differentiation of 11q13 rearrangements from trisomy 11. Moreover, t(11;14) with breakpoints centromeric to MTC cannot be detected with this strategy.

In order to detect t(11;14) simply and reliably, we developed an interphase FISH method by using a commercially available large 11q13 probe and an *IGH*-specific BAC probe. We chose this latter probe because of its large size (>100 kb), enabling unerring detection on interphase cells (Fig. 2). This set of probes gave clear, bright signals in every case. We found an *IGH–CCND1* fusion in 17 of 17 patients with a documented typical MCL. This 100% positivity demonstrates the high diagnostic sensitivity of this set of probes, which compares favorably with other diagnostic techniques such as cytogenetics, Southern blot, or PCR. In this series, cytogenetics was performed on only three patients (because routine cytogenetics is not performed for lymphomas in our institution). Interestingly, one of these three patients with a normal karyotype displayed a fusion by using FISH, reflecting difficulties to interpret cytogenetic results when no abnormality is found. Southern blotting was performed on six patients, showing a rearrangement in three cases. FISH showed a fusion in these three patients, but also in two others. This discrepancy is explained by the diversity of breakpoints on chromosome 11. We

did not perform PCR, but several previous studies showed that only about 50% of true MCL patients are diagnosed by PCR, because of the wide dispersion of breakpoints on chromosome 11 (de Boer et al., 1993; Rimokh et al., 1994; Luthra et al., 1995).

Moreover, this FISH technique is very easy to perform and gives an accurate assessment in less than 24 hr. Interestingly, two patients with a histologically proven MCL did not overexpress cyclin D1, as shown by the negative Northern blot. The RNA amount and quality were carefully assessed. In these two patients, Northern analysis with a control *GAPDH* probe showed the presence of correct amounts of undegraded RNA. Moreover, FISH analysis showed an *IGH–CCND1* fusion of type A configuration; therefore, it did not differ from other MCL cases. These two cases are true t(11;14)-positive MCL patients with absence of *CCND1* overexpression. One possible explanation could be an 11q13 breakpoint located downstream of the *CCND1* gene, but still splitting the probe.

We also found such a fusion in 11 of 21 patients with an MCL-type immunophenotype, but with absent or discordant histology. These patients have either a leukemic MCL or an atypical CLL with t(11;14). Atypical CLL probably represents a collection of different entities that should be separated. Genetic abnormalities such as t(11;14) may be common denominators to individualize new pathological subgroups. Moreover, both MCL and atypical CLL with t(11;14) have a very poor prognosis and could be grouped together as "mantle cell leukemia," as proposed by Neilson et al. (1996). Thus, the demonstration of the *IGH–CCND1* fusion is essential in atypical CLL. Immunophenotyping is clearly not a good technique for this purpose, because half of the cases with a typical MCL phenotype did not share the typical gene fusion. This FISH technique could be performed systematically in patients with CLL and an MCL-type immunophenotype, in order to better characterize this type of B-cell malignancy, as previously proposed (Hernandez et al., 1995; Cuneo et al., 1997).

In conclusion, interphase FISH using this set of large probes allows a rapid and reliable assessment of t(11;14). Such a set of probes would enable large-scale studies for better definition of the incidence and prognostic significance of the *IGH–CCND1* fusion in B-cell malignancies. Of special interest is that it would allow for a better nosologic definition of the so-called atypical CLL: the forms with an *IGH–CCND1* fusion might be considered as MCL, and the forms without such a fusion could be considered as typical CLL.

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