

Detection of Translocation t(11;14)(q13;q32) in Mantle Cell Lymphoma by Fluorescence *in Situ* Hybridization

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To assess an unequivocal diagnosis of mantle cell lymphoma (MCL), we have developed a fluorescence *in situ* hybridization (FISH) assay, enabling the demonstration of t(11;14)(q13;q32) directly on pathological samples. We have first selected *CCND1* and *IGH* probes encompassing the breakpoint regions on both chromosomes. Then, we have defined experimental conditions enabling us to obtain bright clear-cut signals in all of the samples, independently of the initial fixation conditions. We have analyzed single-cell suspensions from 26 formalin-fixed, paraffin-embedded MCL samples with this set of probes. In all cases, we have found a fusion signal (ie, a t(11;14)(q13;q32) translocation) in 14% to 99% of cells (median, 87%). So far, *IGH-CCND1* fusions have been detected in all of the 51 MCL patients that we have analyzed by FISH (either on paraffin-embedded tumor samples or on peripheral blood samples). Regarding the low sensitivity of other techniques used to diagnose t(11;14)(q13;q32) (ie, 70% to 75% for cytogenetics and 50% to 60% for polymerase chain reaction), our FISH assay is by far the most sensitive technique. Moreover, because of the quality of the fluorescent signals and the rapidity of the experiment, this technique is widely applicable, even in routine cytogenetics or pathology laboratories. As MCL patients are usually refractory to standard therapy, an unambiguous diagnosis is needed to propose adapted therapeutic strategies, and this highly sensitive assay may be of great value for accurate diagnosis in difficult cases. (*Am J Pathol* 1999, 154:1449–1452)

logical and immunological criteria, leading to the definition of several NHL subsets. One of this newly identified subgroup is the so-called mantle cell lymphoma (MCL), defined by CD5-positive/CD23-negative/sIgMD-positive lymphoid cells.^{1–5} These cells are the malignant counterpart of naive B cells located in the mantle zone of secondary follicles. Besides these biological features, MCL is characterized by a male predominance, frequent advanced clinical stage at diagnosis, and poor prognosis.^{5–8} This clinical aggressivity contrasts with the intermediate pathological grade, and reliable sensitive diagnostic tools would be highly useful for an unequivocal identification of this NHL subset.

Cytogenetic analyses have revealed that MCL is closely associated with the t(11;14)(q13;q32).^{9–11} This translocation juxtaposes Ig heavy chain gene (*IGH*) sequences with the *BCL-1* locus, leading to up-regulation of the *CCND1* gene and consequently to an overexpression of cyclin D1.^{12–15} However, whereas overexpression of cyclin D1 is thought to be present in 100% of patients with MCL, t(11;14)(q13;q32) is found in 70% to 75% of the patients only.^{10,11} This discrepancy may be related to the low mitotic index of malignant cells and to the poor morphology of metaphase spreads. Moreover, cytogenetic analysis is a time-consuming technique, especially for the analysis of lymphoma samples. Other molecular techniques have also their own limitations, essentially because of the scattering of 11q13 breakpoints. Thus, polymerase chain reaction (PCR) techniques are positive in only 50% to 60% of cases.^{12,16,17} Fluorescence *in situ* hybridization (FISH) may circumvent these difficulties, and we have previously demonstrated that this technique could be successfully applied on MCL leukemic phase samples.¹⁸ However, this approach is applicable only to bone marrow or peripheral blood specimens, or to specimens prepared for cytogenetic analysis, and so cannot be used in patients with suspected MCL but without significant marrow involvement. To circumvent this limitation, we have developed and tested a dual-color FISH assay that could be performed directly on formalin-fixed, paraffin-embedded specimens.

Classification of non-Hodgkin's lymphoma (NHL) is essentially based on morphological features of malignant cells. A more recent classification, ie, the revised European-American classification of lymphoid neoplasms,¹ is probably the most accurate, taking into account morpho-

J.-Y. Li is a grant recipient from the Conseil Régional des Pays de Loire.

Accepted for publication February 18, 1999.

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Table 1. Patient Characteristics

Patient	Sex	Age	IHC results*	% cells with t(11;14)	11q13 probe split†
1	M	56	+	95	Y
2	F	63	-	14	Y
3	M	70	+	99	Y
4	M	61	+	98	Y
5	M	54	Not tested	98	Y
6	M	67	+	92	Y
7	M	77	+	76	Y
8	M	61	+	52	Y
9	M	40	+	98	Y
10	M	64	+	91	N
11	F	68	+	95	Y
12	M	46	+	21	N
13	F	72	+	87	N
14	F	77	+	43	Y
15	M	62	+	76	Y
16	M	57	+	92	Y
17	M	78	+	68	N
18	M	65	+	76	Y
19	M	65	Not tested	63	Y
20	M	41	+	66	Y
21	F	70	+	92	Y
22	F	45	+	79	Y
23	M	71	+	99	Y
24	M	69	+	95	Y
25	F	68	+	87	Y
26	F	73	+	74	Y

M, male; F, female.

*Results of immunohistochemistry (IHC) using a cyclin D1 monoclonal antibody. +, positivity; -, absence of signal with this antibody.

†Y indicates a split of the 11q13 probe, whereas N indicates absence of detectable split.

Patients, Materials, and Methods

Patients

Twenty-six patients diagnosed as MCL (pan-B⁺, CD5⁺, IgMD⁺, CD23⁻) in our institution and for whom a paraffin-embedded sample was available were analyzed. The main clinical features of these patients are described in Table 1. Briefly, they were 18 males and 8 females, with a median age of 65 (range, 40 to 78) years. Five patients with a peripheral blood involvement have been previously reported.¹⁸ Twenty-four of these patients had been analyzed by immunohistochemistry, using a cyclin D1 monoclonal antibody (Immunotech, Marseille, France). All but one (patient 2) were positive. Routine cytogenetic or molecular analyses were not performed for any of these patients.

Fluorescence in Situ Hybridization

The 14q32 (BAC 158A2) and 11q13 (cyclin D1 probe, Vysis, Downers Grove, IL) probes have been previously reported.¹⁸ Briefly, the 14q32 probe maps to the JH and first constant regions of the *IGH* gene and was directly labeled with fluorescein isothiocyanate (green). The 11q13 probe was purchased from Vysis and was labeled with SpectrumOrange (orange). FISH was performed according to routine protocols. Briefly, 60 ng of 158A2 probe and 1 μ l of the Vysis probe were mixed with 2 μ g

of Cot-1 DNA (Life Technologies, Gaithersburg, MD) in 10 μ l of Hybrisol VII (Oncor, Gaithersburg, MD), denatured at 73°C for 10 minutes, and dropped on slides previously denatured at 73°C for 5 minutes. After overnight hybridization at 37°C, slides were washed in 2X SSC at 73°C for 5 minutes and rinsed in 2X SSC/0.1% Triton. Nuclei were then counterstained with 4',6-diamidino-2-phenylindole in antifade, and at least 200 nuclei were examined using an epifluorescence microscope (Axioplan 2, Zeiss, Jena, Germany) equipped with a cooled CCD camera (Photometrics, Tucson, AZ) and appropriate filters specific for fluorescein isothiocyanate, SpectrumOrange, and DAPI (Chroma, Brattleboro, VT). Images were then captured using the SmartCapture VP software (Vysis).

Sample Preparation

In a first attempt, we performed FISH on thin sections (3 to 5 μ m). Despite a good hybridization efficiency, analysis was limited by the small size and the high density of cells. Consequently, determination of the exact location of FISH signals within each nucleus was extremely difficult, and the method was abandoned. To perform a cell-by-cell analysis, we decided to include a tissue disaggregation step to obtain cell suspension. After dewaxing in xylene, 30- μ m-thick slices were incubated for 30 minutes to 2 hours in 0.5% to 5% pepsin, pH 1.5, at 37°C. After a rapid wash in PBS, cells were incubated for 30 minutes in 0.075 mol/L KCl and fixed in methanol/acetic acid (3/1 v/v) and dropped on slides.

Results

Fluorescence in Situ Hybridization Analysis

We have previously shown that the t(11;14)(q13;q32) translocation leads to a split of the 11q13 probe in most patients (25/28) analyzed on bone marrow or peripheral blood samples. In this major configuration, t(11;14)(q13;q32) was assessed in case of the presence of a YGRR configuration (Figure 1A): one yellow signal (fusion on the derivative chromosome 14), one green signal (normal 14), and two red signals (on both normal and derivative chromosome 11). Another possible configuration was YGR (Figure 1B): in this case, the red signal on the derivative chromosome 11 is not observed, either because the size of the probe that hybridizes on this der(11) is too small or because the breakpoint occurs upstream from the probe without any split. In contrast, on chromosome 14, breakpoints are clustered in the *JH* region, and in t(11;14)(q13;q32)-positive cases, almost the whole 158A2 probe remains on the der(14).

Different tissue digestion conditions were tested to obtain reproducible FISH results. We have tested different pepsin concentrations and different incubation times. The best results were obtained with incubation in 0.5% pepsin for 30 minutes. In these conditions, we achieved a good FISH efficiency in almost all of the cases. To determine the t(11;14)(q13;q32) positivity cut-off with these conditions, we analyzed other NHL subset specimens

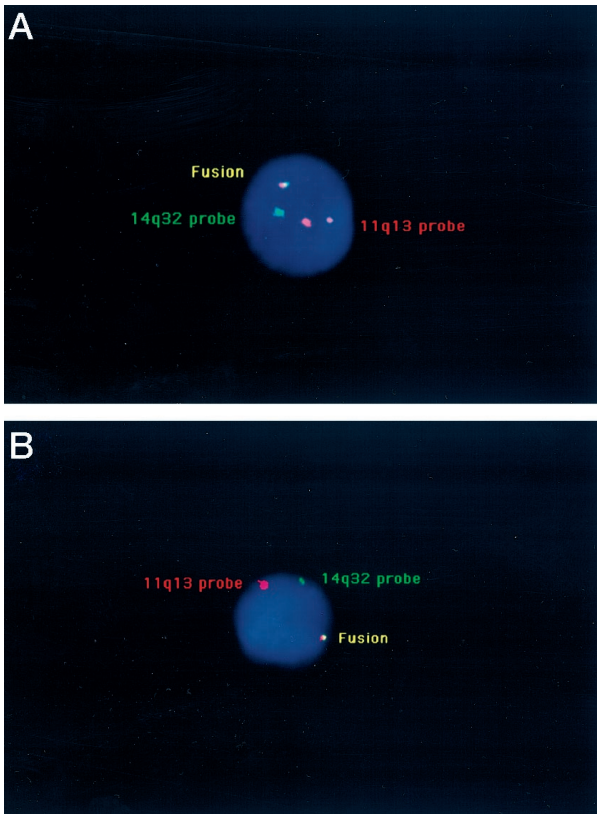


Figure 1. **A:** A malignant cell with *IGH-CCND1* fusion and a split of the 11q13 probe (major configuration). The two red/orange signals correspond to the 11q13 probe, hybridized on the normal and derivative chromosomes 11, respectively. The green signal corresponds to the normal chromosome 14, whereas the yellow signal corresponds to the fusion of the 11q13 and 14q32 probes on the derivative chromosome 14. **B:** A malignant cell with an *IGH-CCND1* fusion, but lacking a split of the 11q13 probe (minor configuration). Only one red/orange signal corresponding to the normal chromosome 11 is observed.

prepared in the same way (ie, from formalin-fixed, paraffin-embedded cells). We selected six B-cell diffuse large-cell lymphoma and six Burkitt's lymphoma samples and blindly scored 300 nuclei in each case (total of 3600 nuclei). A nucleus was defined as t(11;14)(q13;q32) positive if a yellow fusion signal was observed, independently of the presence of a split of the 11q13 probe. In these controls, 2% to 5.7% (mean, 3.11; SD = 1.01%) of nuclei displayed a co-localization of a green and an orange signal. Thus, the cut-off level (mean + 3SD) for the detection of the t(11;14)(q13;q32) was set at 7%.

A t(11;14) Is Found by FISH in 26/26 Patients

In all 26 MCL samples investigated, more than 10% of nuclei displayed a fusion between 11q13 and 14q32 sequences. The percentage of cells with at least one co-localization was variable, ranging from 14% to 99% (median, 87%). In two patients (patients 2 and 12), we have observed *IGH-CCND1* fusion only in a minor cell subset (14% and 21%, respectively). This apparent low tumor cell infiltration was in agreement with a marked lymphocytic reaction within the sample in both cases. The patient with 14% t(11;14)(q13;q32)-positive cells

was the only case with negative immunohistochemistry with the cyclin D1 antibody. A split of the 11q13 probe (with a residual signal on the derivative chromosome 11) was observed in 22/26 cases.

Discussion

Mantle cell lymphoma is a recently identified subset of NHL, characterized by a male predominance, a high incidence of disseminated disease at diagnosis, and a poor outcome, even with intensive polychemotherapy regimens.⁴⁻⁸ Incidence of this NHL subtype is variable in published series, but it probably represents 5% to 7% of all NHL cases. A recent retrospective study identified MCL in 121/1919 consecutive cases (6.8%).⁴ Because of the poor prognosis of this NHL subgroup, its accurate identification is extremely important to propose specific intensive therapeutic strategies. Thus, detection of the molecular genetic hallmark of this NHL subtype would definitely help to propose the accurate diagnosis.

So far, several approaches have been reported, none of them fulfilling the requested criteria for an unequivocal diagnosis. The t(11;14)(q13;q32) is found in most but not all MCL cases, and up to 30% of patients with a characteristic MCL lack this translocation.^{10,11} Likewise, molecular detection of the t(11;14)(q13;q32), using either Southern blotting or PCR, is hampered by the scattering of 11q13 breakpoints. As the functional consequence of the translocation is an overexpression of CCND1, the detection of high levels of RNA and/or protein would represent the ideal test. However, so far, no monoclonal antibody fulfills the specificity and sensitivity criteria for an undoubtful diagnosis. Currently, Northern blotting represents the gold standard for detection of CCND1 overexpression. However, this technique requires large amounts of RNA and is not adapted to routine diagnosis.

Several attempts to use FISH for identification of the t(11;14)(q13;q32) have been recently reported.¹⁸⁻²³ Even though the first reports were limited by a low sensitivity, the most recent studies, including ours,¹⁸ based on dual-color FISH techniques, achieved this goal and demonstrated that virtually 100% of MCL patients displayed an *IGH-CCND1* fusion. However, these series analyzed either patients for whom metaphase spreads were available or patients with a high percentage of malignant cells within bone marrow and/or peripheral blood. These studies had the ability to demonstrate the feasibility and reliability of this technique but were limited to a subset of MCL patients. Because NHL cytogenetic analyses are not performed in most routine cytogenetics laboratories, and as not all patients have a massive bone marrow involvement, many patients still escape from this evaluation. To analyze 100% of patients with a MCL diagnosis, we developed a FISH assay that could be performed directly on formalin-fixed, paraffin-embedded specimens.

An ideal experiment would be to perform FISH directly on thin sections, to combine morphological and genetic analyses. Even though we were successful in obtaining efficient hybridization, analysis was hampered by the small size and the high density of malignant cells, pre-

venting us from an accurate cell-by-cell analysis. To circumvent this problem, we set up tissue digestion conditions, enabling the obtainment of a cell suspension while preserving a good cell morphology. Then, we progressively improved experimental conditions to obtain a high hybridization efficiency, even in formalin-fixed, paraffin-embedded cells. We were able to define FISH conditions applicable to all cases. Thus, we have detected *IGH-CCND1* fusion in all 26 analyzed cases. In our previous study on peripheral blood or bone marrow samples, we have shown that *IGH-CCND1* fusion was observed in 17/17 MCL patients.¹⁸ Since this publication, we had the opportunity of analyzing 13 more patients with MCL and peripheral blood involvement. All of these 13 patients displayed an *IGH-CCND1* fusion (unpublished data). So far, the combination of our analyses either on primary tumor or peripheral blood/bone marrow samples shows that an *IGH-CCND1* fusion is observed in 51/51 patients with MCL. As cytogenetics was not performed in these cases, a direct correlation between *IGH-CCND1* fusion and t(11;14)(q13;q32) cannot be definitively assessed. However, this FISH assay enables the detection of every *IGH-CCND1* rearrangement and thus is probably more sensitive than cytogenetics for the detection of cryptic rearrangements.

Thus, we think that we achieved the final goal of a highly sensitive, simple, and rapid technique, usable in 100% of patients, enabling an unambiguous MCL diagnosis. Moreover, as we used one commercially available probe and as the second probe gave bright clear signals without immunological amplification steps, we think that this set of probes could be used in most (if not all) routine cytogenetics (and probably pathology) laboratories.

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