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The Detection of t(14;18) in Archival Lymph Nodes

Development of a Fluorescence in Situ *Hybridization (FISH)-Based Method and Evaluation by Comparison with Polymerase Chain Reaction*

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Fluorescence *in situ* hybridization (FISH) has been used to demonstrate the t(14;18) in up to 100% of follicular lymphoma (FL) cases, however, there is little reproducible data using fixed tissue. The aim was therefore to develop a robust FISH method for the demonstration of translocations in archival tissue. The technique was evaluated by comparison with multiplex polymerase chain reaction (PCR), capable of detecting the majority of known breakpoints. Twenty-eight paired frozen and fixed cases of FL and 20 reactive controls were analyzed. The t(14;18) was detected in 23 of 28 cases using PCR on frozen material and 8 of 20 in paraffin. Using FISH, 24 of 26 frozen and 26 of 28 paraffin cases had a demonstrable translocation. All 20 reactive nodes were negative for the t(14;18) by PCR. Using FISH, one of the reactive cases had occasional cells with a translocation FISH pattern, demonstrable in frozen and paraffin samples. This is consistent with the presence of the t(14;18), which is well described in normal individuals. Both PCR and FISH are highly effective for t(14;18) analysis in unfixed tissue. When only paraffin blocks are available, FISH is the method of choice, and a result was achieved in 100% of cases. The method is applicable to the retrospective analysis of a range of translocations. *(J Mol Diagn 2003, 5:168–175)*

Follicular lymphoma (FL) is characterized by the presence of the t(14;18)(q32;q21) chromosomal translocation, which results in the rearrangement and up-regulation of the *BCL2* proto-oncogene. The t(14;18) has traditionally been detected using cytogenetic assay or Southern blot analysis, with a reported incidence in follicular lymphoma of around 60 to 80%.[1–3] More recently, polymerase chain reaction (PCR) has been used, but highly variable assays have resulted in inconsistent results.[4] Between 40 and 70% of breakpoints can be demonstrated by major breakpoint region (MBR) PCR, and 5 to 10% using minor cluster region (mcr) primers.[5–10] The remaining breakpoints are located 5′ of the *BCL2* gene[11] and in the 20-kb region between the MBR and mcr.[12–15] Long-distance (LD) PCR[6,14–16] strategies have been used to identify breakpoints between the MBR and mcr subcluster regions. Positioned 4 kb downstream of the MBR is a further breakpoint region, the 3′MBR subcluster, encompassing a region of 3.8 kb,[12] and 10 kb upstream of the mcr is the 5′ mcr subcluster.[17,18] LD-PCR techniques are not applicable to routine use, however, for an efficient PCR detection strategy all of these breakpoint regions need to be taken into account. The PCR strategy used in this study is a highly specific multiplex technique capable of detecting the majority of known breakpoints, including MBR, mcr, 3′MBR, and 5′mcr breakpoints and has been validated by the European BIOMED Group.[17,18]

The first reported use of fluorescence *in situ* hybridization (FISH)-based techniques for the demonstration of the t(14;18), were on cytogenetic samples and involved the demonstration of a break of the signal at 14q32, using chromosome paints,[19] a YAC containing the entire *IgH* locus,[20] or a dual color *IgH* break-apart FISH assay.[21] The t(14;18) has been detected in 100% of FLs using a FISH assay based on co-localization of YACs spanning the *BCL2* and *IgH* genes[22] and a *BCL2* break-apart interphase FISH assay, validated by comparison with fiber FISH[23,24] In the present study, the Vysis LSI *IGH/BCL2* probe set was used. This has the advantage over alternative FISH strategies in that it utilizes both probe splitting and co-localization, minimizing the risk of false-positives. Using this approach, the t(14;18) has been detected in 25 of 39 (64%)[25]and 63 of 63 (100%)[26] FLs.

For retrospective studies it is vital that molecular techniques used for the detection of translocations are applicable to paraffin-embedded tissue. Although PCR has

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been used successfully on paraffin-embedded tissue,[6,27–31] the detection rate of the t(14;18) is significantly reduced due to poor quality of DNA. The application of FISH techniques for the detection of chromosomal translocations in paraffin tissue has been less well used, and the methodology is not well described and highly variable. The majority of studies have involved either whole chromosome paints or centromeric probes.[32–35] Locus-specific probes have been used in paraffin material for the demonstration of the Philadelphia chromosome,[36,37] p53 abnormalities,[38] cERB2 and *cMYC* amplification in gastric tumors,[39,40] the t(11;14) in mantle cell lymphoma,[41,42] the t(14;18) in diffuse large B-cell lymphoma,[43] and more recently FISH on nuclei extracted from cores of tissue taken from paraffin blocks has been used to demonstrate a range of abnormalities.[44]

The aim of the study was to develop a relatively simple and reproducible FISH method for the demonstration of chromosomal translocations in archival formalin-fixed, paraffin-embedded tissue. The technique we describe has been evaluated by comparison with paired frozen samples, and with a highly sensitive PCR strategy in the same-paired samples.

Materials and Methods

Twenty-eight histologically defined cases of FL were used in the study. Cases were chosen based on the availability of paired frozen and paraffin-embedded samples. All cases were presentation lymph node biopsies of previously untreated patients. Twenty reactive lymph nodes were used as controls. All paraffin-embedded samples were fixed in 10% formalin and routinely processed.

Multiplex PCR Analysis of the t(14;18)

DNA was extracted from the paired frozen and paraffin-embedded samples in all cases. Amplification of a control gene (β–*Globin*, 320 bp) was used to assess the quality of the DNA from all frozen and formalin-fixed samples. If samples were not amplifiable initially, the control PCR was repeated on a 1:10 dilution of the DNA sample, to minimize the effect of inhibitors within the sample. Based on previous experience (data not presented), samples (neat or a 1:10 dilution) that failed to amplify to at least 320 bp were considered unsuitable for further PCR analysis. This has also been confirmed by the findings of the Biomed group, who showed that amplification of a 400-bp control gene generally indicated a sample that was suitable for further analysis (work carried out by Dr. H. White[17]). Twenty-eight of 28 of the frozen samples and 20 of 28 of the fixed samples were suitable for t(14;18) analysis. A single-round multiplex PCR technique was performed on all samples using the European Biomed-2 concerted action t(14;18) primers and protocols.[17,18] Briefly, two multiplex reactions were used. These included an MBR multiplex strategy and a mcr/3′ MBR/5′ mcr multiplex, used in conjunction with a consensus JH primer. Positive, negative, and no template controls were run in all experiments. The sensitivity of the multiplex reactions was evaluated by amplifying DNA dilutions of the cell lines DoHH2 (MBR +ve), SC-1 (mcr +ve), Oz (5′mcr), and K231 (3′MBR) in normal tonsil DNA. A sensitivity of between 3.3×10^{-2} and 10^{-3} was consistently achievable using both multiplex reactions (data not shown).

All samples were analyzed on at least two occasions. Amplification was performed in an automated thermal cycler (Geneamp 9700 or 2700 PCR system; PE Biosystems, Foster City, CA) and was identical for each multiplex reaction. Each 50-μl reaction was performed using 100 ng genomic DNA in 1X reaction buffer containing 1.5 mmol/L $MgCl_2$ (Amplitaq Gold Buffer II, PE Biosystems), 0.125 mmol/L dNTP's (Pharmacia Biotech, Amersham, England), 10 pmols of each primer, and 1 unit of *Taq* polymerase (Amplitaq Gold, PE Biosystems). Amplification was performed as follows: initial denaturation/enzyme activation at 94°C for 10 minutes; followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute; followed by a final extension of 72°C for 10 minutes and rapid cooling to 4°C. Products were resolved on 2% agarose gels and visualized under ultraviolet illumination with ethidium bromide staining.

FISH Analysis

FISH for the t(14;18) was performed on both the frozen and paraffin-embedded samples on all FL and reactive cases using the Vysis LSI *IgH* Spectrum Green/LSI *BCL2* Spectrum Orange probe set (Vysis Inc., No. 32–191018), which includes the probe and the hybridization buffer.

FISH on Touch Preparations from Frozen Tissue

The lymph nodes were taken from the store, allowed to defrost, and a microscope slide was gently touched onto the tissue surface to make an impression. FISH was performed after allowing the slides to air dry.

FISH on Whole Nuclei Extracted from Paraffin-Embedded Tissue

This method was developed by the systematic evaluation of various methodologies, pre-treatments, and enzymes (data not shown). The chosen method, first described for the flow cytometric analysis of ploidy and S-phase,[45] was found to give the most consistent results with a range of paraffin tissue from various sources and with diverse fixation and processing protocols (results not shown).

Between 5 and 10 (depending on the size of the tissue in the block) 35-μm thick paraffin sections were cut, dewaxed in xylene at 37°C, and rehydrated through graded alcohols. Following two changes of distilled water, the sections were incubated in pre-warmed digestion buffer (0.1 mol/L Tris; 0.07 mol/L NaCl; 0.1% NP-40; pH 7.4) at 37°C for 30 minutes. The digestion buffer was

replaced with fresh, pre-warmed buffer containing 0.05% Protease XXIV (Sigma P8038). The sections were agitated for 1 hour at 37°C. All processing steps were carried out in 10-ml glass or xylene resistant plastic specimen tubes and the waste solvents were changed by careful pipetting. Digested nuclei were harvested by pipetting the supernatant nuclear suspension from the undigested tissue, and centrifuging at 1800 × *g*. The harvested nuclei were washed once in phosphate-buffered saline (PBS) and once in 3:1 methanol acetic acid (MAA). Centrifugation at each stage was at 1800 × *g*. The nuclei were resuspended in MAA, and the nuclear suspension was dropped by pipette onto 3-aminopropyltriethoxy-silane (APES)-coated microscope slides. An optimum concentration resulted in an evenly spread monolayer of nuclei on the slide. The concentration and morphology of the nuclei were checked by staining a slide with May Grunwold Giemsa before commencing FISH.

FISH Method

FISH for the t(14;18) was carried out according to the manufacturer's instructions. Briefly, the slides were fixed in MAA for at least 30 minutes, and incubated in 2X standard saline citrate (SSC) at 37°C for at least 1 hour. Following dehydration through graded alcohols, the probe was applied in hybridization buffer, and a coverslip was sealed on using rubber cement.

Denaturation/Hybridization

An additional pre-denaturation step was used for the paraffin nuclear preparations. The slides were incubated in hybridization buffer at 85 to 90°C for 2 to 20 minutes, and then allowed to cool back to 37°C. (The pre-denaturation time and temperature can be adjusted accordingly to obtain optimal hybridization efficiency). Using a Vysis Hybrite machine, denaturation was 73°C for 3 minutes and 75°C for 5 minutes for the frozen and paraffin preparations, respectively, and hybridization was at 37°C for 16 to 24 hours.

Post-Hybridization Washes

Post-hybridization washes consisted of 2 × 2 minutes in pre-warmed 0.4XSSC/0.3XNP-40 at 70°C; followed by 5 minutes in 2X SSC/0.1X NP-40 at room temperature. 4′-6-diamino-2-phenylindole (DAPI) was used as the counterstain.

Interpretation of FISH

Representative images were captured using Metasystems ISIS software. Cases were defined as normal if there were two green (*IgH*) and two red (*BCL2*) signals (Figure 1a). A number of cases showed a significant proportion of nuclei in which one each of the signals was co-localizing. In the absence of any extra signals these cases were also classed as normal.

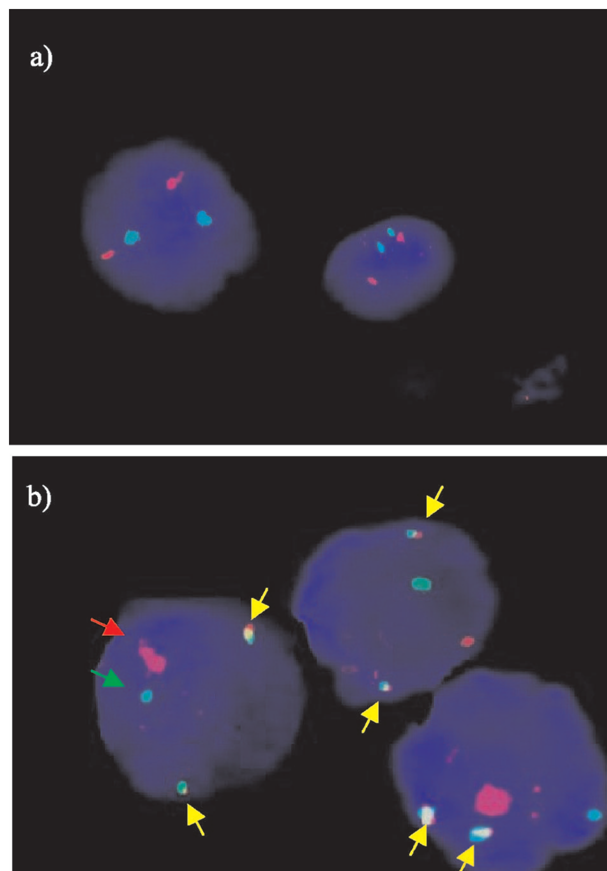

Figure 1. Representative FISH images of the pattern of staining of the BCL2/IgH probe set. **a:** Normal pattern of staining. Each cell nucleus has two red (*BCL2*) and two green (*IgH*) FISH signals, one for each copy of the chromosome. The counterstain is DAPI, which is visualized as blue fluorescence. **b:** A case with a t(14;18). Each nucleus has three signals with both *BCL2* and *IgH* probe sets, indicating a "split" in one each of the copies of the genes. The reciprocal translocation, t(14;18), is demonstrated by the presence of two fusion signals in each cell (indicated by yellow **arrows**), along with a residual normal copy of each gene. The counterstain is DAPI.

A t(14;18) was defined when there were extra signals of both the *BCL2* and the *IgH* probes, indicative of a chromosomal break, along with at least two of the extra signals co-localizing to produce a yellow fusion signal (Figure 1b), as described in the product datasheet. Extra signals of either *BCL2* or *IgH* in the absence of co-localization would be suggestive of either an alternative translocation partner or extra copies of the gene or chromosome.

The entire preparation was analyzed and at least 100 nuclei were formally counted in each case. In general, a cut-off of 5% was used to define a t(14;18)-positive case. If <5% of cells had the classical translocation pattern of signals, up to 500 additional nuclei were counted. Given the stringent criteria used to define the presence of the translocation within a cell, a threshold as low as one cell per preparation is sufficient to class a case as positive, since it is not possible for the translocation pattern of signals to occur by chance.

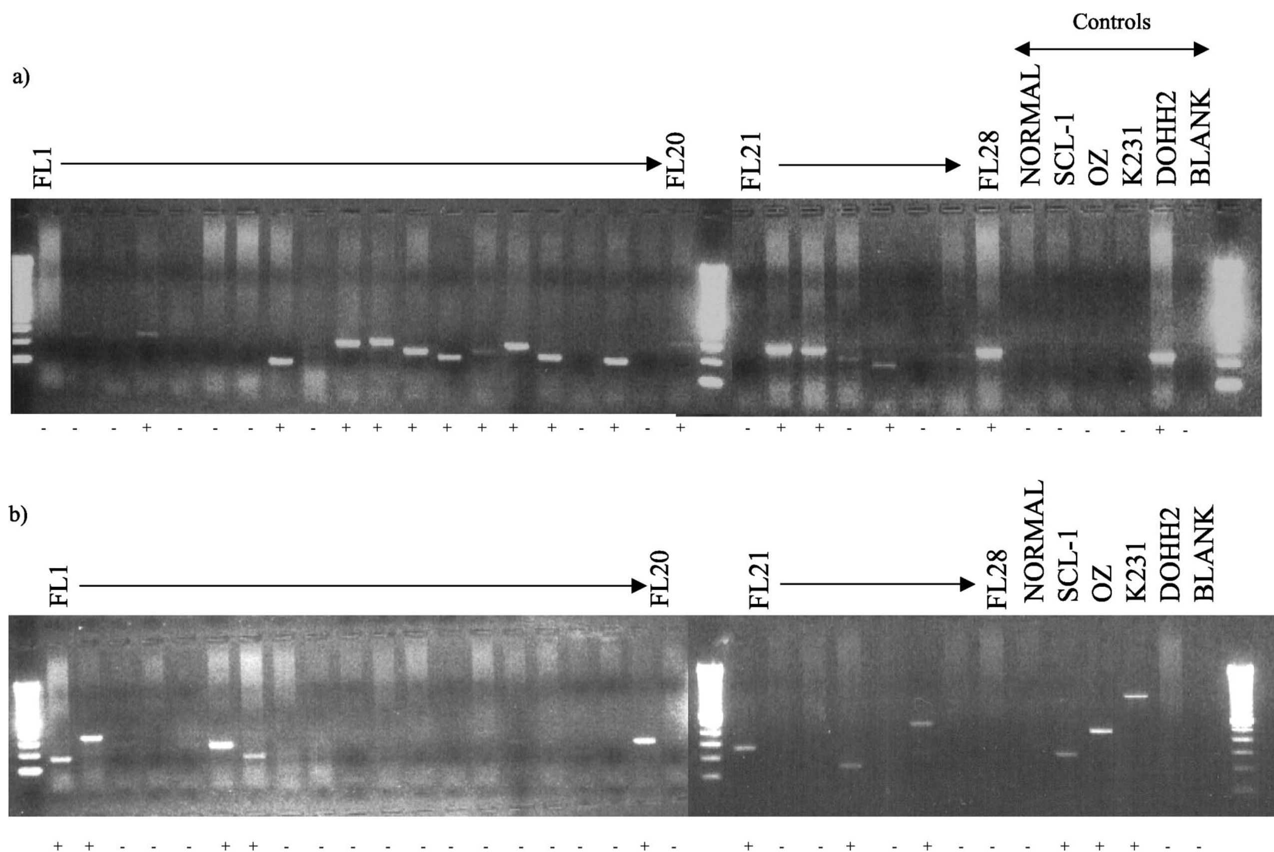

Figure 2. t(14;18) multiplex PCR analysis on DNA extracted from 28 frozen biopsies of FL. **a:** MBR multiplex (JH + MBR): 15 of 28 FL cases had a visible band and were classed as positive (+). **b:** mcr multiplex (JH + mcr, 5′mcr, 3′MBR): 8 of 28 FL cases had a visible band and were classed as positive (+).

Results

We were able to detect the translocation in 23 of 28 cases using PCR on frozen material, breakpoints were detected at the MBR in 16 of 23 (70%), mcr in 5 of 23 (21%), and 5′mcr in 2 of 23 (9%). No cases were demonstrated with a breakpoint in the 3′MBR region. Figure 2 shows the agarose gel electrophoresis of the MBR and mcr multiplex PCR products from DNA extracted from 28 frozen FL lymph nodes. Eight of 20 (40%) cases with amplifiable DNA were positive using PCR on paraffin material. Using FISH, 24 of 26 cases were positive on the frozen touch preparations, and 26 of 28 were positive on the paraffin nuclei. In five cases the efficiency of hybridization was low, however occasional cells (<5%) were seen with the classical translocation pattern of signals, ie, three red, three green signals with two fusion signals, and a residual red and green signal. All five cases showed concordance between the frozen and paraffin preparations and in 3 of 5 of these cases, the t(14;18) was demonstrated using PCR. These cases were therefore classified as t(14;18)-positive. No cases were positive by PCR but negative by FISH. The results are detailed in Table 1.

The concordance of results between the frozen and paraffin-embedded samples was 100% with the FISH method, compared to 60% by PCR. Eight of the 20 cases with amplifiable paraffin-extracted DNA gave a false-negative result by comparison with the PCR on DNA extracted from frozen tissue.

Analysis of Reactive Lymph Nodes

Amplifiable DNA was produced in all reactive lymph nodes in both the frozen and paraffin-embedded paired samples. All cases were negative for the t(14;18) by PCR. Using the FISH method, 1 of 20 of the reactive cases had occasional cells (<5%) with the classical translocation pattern of signals, ie, two co-localized signals along with a residual red and green signal. This was demonstrated in both the frozen touch preparation and the paraffin-extracted nuclei.

Discussion

In this study we have described an effective FISH method, using the Vysis LSI *IGH/BCL2* probe set, for the demonstration of the t(14;18) on archival material. The probe set consists of a 1.5 Mb locus-specific *IgH* probe spanning the entire *IgH* locus and extending 300 kb beyond the 3′ end, and a 750 kb *BCL2* probe spanning the entire *BCL2* gene and extending 250 kb both distal and proximal to the gene, labeled Spectrum Green and Spectrum Orange, respectively. This has the advantage over previous FISH strategies in that it utilizes both probe splitting and co-localization. These stringent criteria used to define the translocation have the effect of minimizing false-positives that may occur as a result of random

Table 1. Detection of the t(14;18) in 28 Cases of Frozen and Paffin Samples of FL, Comparing the Effectiveness of PCR to FISH

Case no.	Amplifable DNA (DNA dilution factor)		PCR for t(14;18)		FISH for t(14;18)	
	Frozen	Paraffin	Frozen	Paraffin	Frozen	Paraffin
1	+	+ (1/10)	MCR +	−	+	++
2	+	−	5′ MCR +	NT	Failed	+
3	+	+ (1/10)	−	−	+	+
4	+	+ (1/10)	MBR +	−	+++	+++
5	+	+ (1/10)	−	−	−	−
6	+	+ (1/10)	MCR +	+	++	++
7	+	+	+	−	++	+
8	+	−	MBR +	NT	++	+
9	+	−	−	NT	−	−
10	+	+ (1/10)	MBR +	+	++	+
11	+	+	MBR +	−	+	++
12	+	+ (1/10)	MBR +	+	+	+
13	+	+	MBR +	+	+++	+++
14	+	+	MBR +	−	++	+
15	+	+	MBR +	+	++	++
16	+	+	MBR +	+	+	+
17	+	+	−	−	++	+
18	+	−	MBR +	NT	++	+
19	+	+ (1/10)	MCR +	+	+++	++
20	+	+	MBR +	−	+	+
21	+	−	MCR +	NT	++	+
22	+	−	MBR +	NT	++	++
23	+	+	MBR +	−	++	+
24	+	+ (1/10)	MCR +	+	++	++
25	+	−	MBR +	NT	Failed	+
26	+	+	5′ MCR +	−	++	++
27	+	+	−	−	+*	+*
28	+	−	MBR +	NT	++	++

*, Case 27 appeared to have a complex translocation pattern by FISH analysis. Occasional cells were seen with the classical translocation pattern, but additional cells with extra *BCL2* signals but no co-localization, and cells with extra *IgH* signals but without co-localization were also observed.

NT, not tested: no amplifyable DNA; MCR, minor cluster region; MBR, major breakpoint region; +, occasional double fusions; ++, majority of cells double fusions; +++, double and triple fusions.

localization of signals, or due to the structural organization of the chromosomes within the nucleus.[46–48]

The method was validated by analysis of the same series of samples using PCR, and by the analysis of paired frozen and paraffin samples using both methods. The PCR strategy used was the method developed and validated by the European Biomed group.[17,18] The multiplex approach is based on the detailed investigation of breakpoints and primer binding and enables the detection of the majority of known breakpoints, making this PCR method a highly effective technique for the detection of the t(14;18).

The detection rate of these primers has also been extensively studied in paraffin-embedded material[17] and it was shown that amplification of a 400-bp control gene indicated that a sample was suitable for further analysis (work carried out by Dr. H. White[17]). Amplifiable DNA was obtained in 100% of the frozen lymph nodes and 71% of paraffin cases, which is superior to previous studies that have reported amplifiable DNA in 60% and 50% of cases, respectively.[31]

In frozen tissue, all cases produced a result by PCR, however two cases failed using FISH, due to insufficient cells on the touch preparation. The t(14;18) was positive in 82% and 92% of FL cases, respectively. In archival formalin-fixed, paraffin wax-embedded tissue, a result was achieved in 100% of cases using the FISH method, compared to 71% using PCR. In paraffin, the t(14;18) was detected in 93% and 40% of cases, respectively. The reported incidence of the t(14;18) as detected by MBR and mcr PCR is highly variable; reported positive, at best, in 82% of FL patients by analysis of fresh material,[9] and 47% by analysis of DNA extracted from paraffin tissue.[28] In contrast, the t(14;18) has been demonstrated in up to 100% of cases of FL using FISH.[23,26]

Concordance of results comparing FISH and PCR was 88% in frozen tissue and 45% in paraffin tissue. There was a 100% correlation using FISH in the paired frozen and paraffin samples and all cases that were positive using frozen tissue PCR were positive using FISH. In contrast, a significant false-negative result was seen using PCR on archival DNA, with 40% of cases giving a false-negative result compared with PCR on frozen tissue.

Using FISH analysis, a proportion of cases had only occasional cells with the translocation. The low percentage of positive cells is most likely to be due to a high percentage of reactive T and B cells in the lymph node sample. Given the stringent criteria used to define the translocation using FISH, it is unlikely that this low frequency of positive cells was an artifact. In contrast, this suggests that the method is highly sensitive, provided that large numbers of cells are counted.

Using PCR, five cases were translocation-negative in the frozen tissue samples, however only two of these

cases were negative using FISH. Of the remaining three cases that were positive by FISH, but negative by PCR, two had only occasional cells positive for the translocation. This highlights a possible sensitivity issue for the PCR technique used in this study, which may help to explain the false-negative results obtained using PCR compared to FISH and in paraffin material compared to the matched frozen tissue. The t(14;18) multiplex developed by the Biomed group is based on the use of a single round PCR amplification, which is sensitive up to 1 in 1000. Previous studies using DNA derived from paraffin-embedded material have used a nested PCR strategy, which has been shown to have an increased sensitivity up to 1000-fold higher than conventional PCR. In addition, PCR requires absolute sequence complementarity that is not as crucial for FISH. In follicular lymphoma, ongoing somatic hypermutation is a key feature and may result in base changes at the primer-binding site, reducing the complementarity. The further case that was positive by FISH but negative by PCR appeared to have a complex translocation pattern. Occasional cells had the classical FISH pattern, but additional cells had extra *BCL2* and/or extra *IgH* signals but without co-localization. This highlights an additional advantage of FISH over PCR for the detection of gene rearrangements, in that PCR will not detect alternative breakpoints that are outside the regions covered by the PCR strategy. Single fusions signals were seen using FISH in a number of cases, and was particularly common in the reactive cases. In the absence of a split of either signal, these cases were classified as translocation-negative. This pattern of signals can be explained simply by the spatial organization of the chromosomes within the nucleus.[46–48] An alternative explanation is the insertion of the entire *BCL2* gene into *IgH,* which has been reported in FL[24] and would also manifest as a single fusion using FISH, however this possibility was not further investigated. The main disadvantage of this FISH method compared to PCR is that no information regarding the breakpoint can be given. However, due to the mapping of the probes, a positive result was observed by FISH analysis regardless of the breakpoint demonstrated by PCR.

The technique was further validated by analysis of a small series of reactive lymph node biopsies. All cases were PCR-negative and 19 of 20 cases gave a negative FISH result in both the paraffin and frozen material. One reactive case showed a very occasional cell with the classical translocation pattern of signals. This was demonstrated in both the paraffin and the frozen tissue, but was not confirmed by PCR. The presence of the t(14;18) translocation has been well described in reactive tissue by PCR[49–52] and FISH,[22] and in the peripheral blood of normal individuals.[53–55] Using TaqMan real-time PCR, 23% of normal individuals were found to be positive for the translocation, with 3% of these at a level of more than 1 in 10,000 cells.[56] As described above, the FISH technique is highly specific for the translocation and is unlikely to producing a false-positive result. The most likely explanation is that the translocation was unable to be detected by PCR for the reasons highlighted above.

Both PCR and FISH are highly effective techniques for the analysis of t(14;18) where unfixed tissue is available. When only paraffin blocks are available, FISH is superior to even this gold standard PCR technique. The main advantage of the method described here is the flexibility it offers for samples that have been fixed and processed using a range of different protocols, which is of particular interest in the clinical trial setting or in a reference laboratory. All cases can be digested using the standard enzyme protocol, and the pre-denaturation time can be adjusted accordingly.

FISH may also have the added advantage over PCR in the analysis of complex translocations. Three cases were demonstrated to have multiple fusion signals by FISH analysis, but there was no difference in these cases by PCR. Although the significance of additional fusion signals is not well understood, it may have prognostic significance. The relative costs of FISH and PCR per test would favor the use of PCR. If cost is a major concern then to minimize expenses, PCR could be used as a "screen" for the presence of the translocation, and FISH only performed on cases that are PCR-negative.

In conclusion, the FISH method described here is a highly sensitive technique for the demonstration of the t(14;18) in archival tissue. The method can be applied to enable the retrospective analysis of a range of translocations, and may be useful in determining the diagnosis in problem cases, for example when FL and marginal zone lymphoma or reactive hyperplasia are the differential diagnoses.

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