Double-staining chromogenic in situ hybridization as a useful alternative to split-signal fluorescence in situ hybridization in lymphoma diagnostics

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Background
Malignant lymphomas are classified based on morphology, immunophenotype, genetics and clinical features. The pathological diagnosis is generally considered difficult and prone to mistakes. Since non-random chromosomal translocations are specifically involved in specific entities, their detection is an important adjunct for increasing the reliability of the diagnosis. Recently, split-signal fluorescence in situ hybridization has become available as a robust method to detect chromosomal breaks in paraffin-embedded formalin-fixed tissues. A bright field approach would bring this technology within the reach of every pathology laboratory.

Design and Methods
Our study was initiated to determine the consistency between chromogenic in situ hybridization and fluorescence in situ hybridization, both using split-signal probes developed for the detection of chromosomal breaks. Five hundred and forty cases of 11 lymphoma entities and reactive, benign lymphoid tissues, collected from eight different pathology laboratories, placed on 15 fluorescence in situ hybridization pre-stained tissue microarray slides, were double stained for the chromogenic hybridization. For each core morphology and actual signal were compared to the original fluorescence hybridization results. In addition, hematoxylin background staining intensity and signal intensity of the double-staining chromogenic in situ hybridization procedure were analyzed.

Results
With respect to the presence or absence of chromosomal breaks, 97% concordance was found between the results of the two techniques. Hematoxylin background staining intensity and signal intensity were found to correspond. The overall morphology after double-staining chromogenic in situ hybridization had decreased compared to the initial morphology scored after split-signal fluorescence in situ hybridization staining.

Conclusions
We conclude that double-staining chromogenic in situ hybridization is equally reliable as fluorescence in situ hybridization in detecting chromosomal breaks in lymphoid tissue. Although differences in morphology, hematoxylin staining and chromogenic signal intensity vary between the tumor entities none of the entities appeared more easy or difficult to score.

Key words: double staining, CISH, split-signal, lymphoma diagnostics.


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Introduction

The World Health Organization (WHO) has classified about 40 different lymphoma entities that differ widely in clinical behavior and therapy response. The diagnosis of lymphomas is a complex process, which needs to take into account clinical, morphological, immunophenotypic and genetic features. As the therapeutic options for lymphoma patients increase, the need for a precise pathobiological diagnosis and classification becomes more important. It is known that different types of lymphoma are associated with non-random chromosomal translocations.\(^{1,2,3,4,5}\) The detection of these aberrations is, therefore, an important step in the identification of specific lymphoma entities. Several techniques are now available to detect chromosomal alterations.

Cytogenetic analysis, based on banding techniques, provides an overview of all cytogenetic aberrations. However, failed culturing of tumor cells, low mitotic indices and the lack of fresh material often complicate the use of this technology for routine diagnosis. Polymerase chain reaction (PCR)-based methods have the advantage of being sensitive and applicable in paraffin-embedded, formalin-fixed tissues, but only for those rare cases in which the chromosomal breaks involved in the translocation are clustered in a small area.\(^6\) In a recent review,\(^7\) it was nicely outlined that fluorescence in situ hybridization (FISH) has, over the last decade, become a firmly established technique and the method of choice in routine clinical practice.

To detect a translocation in a tumor cell one can use fusion probes [probes with different colors on different chromosomes (usually two)] which, in the case of a translocation, show a fusion signal.\(^8\) This procedure is feasible when complete cells or nuclei can be evaluated as in cytospins or preparations of isolated nuclei, but is more difficult in tissue sections, in which many nuclei are cut and/or overlap resulting in the presence of a complete signal in only a minority of cells, making interpretation cumbersome. In order to detect a chromosomal break reliably, it is desirable to be able to analyze tissue sections for translocations, since often only a minority of cells in a biopsy are lymphoma cells making a direct comparison with routine hematoxylin and eosin-stained tissue sections and immunostained slides important. Split-signal or break-apart probes use differently colored probes on both sides of a known breakpoint region, resulting in a fused signal in normal cells, and two different single colors when a chromosomal break occurs.\(^9\) This approach is advantageous in tissue sections since each single colored signal indicates a specific chromosomal break.

Just recently, the Euro-FISH project, representing a concerted multicenter retrospective study in the field of lymphoma diagnosis on paraffin-embedded material, demonstrated the robustness of a FISH protocol.\(^{10}\) In this study we describe double-staining chromogenic in situ hybridization (DuoCISH) as an alternative to split-signal FISH in the diagnosis of lymphoma.

Design and Methods

Materials

Sixty-four split-signal FISH-stained tissue microarray (TMA) slides, cut from four different TMA paraffin blocks, with each block consisting of 36 cores, of paraffin-embedded, neutral-buffered formalin-fixed biopsies were kindly provided by the Euro-FISH consortium.\(^{11}\) Areas of tumor cells used to prepare the TMA were specifically pre-analyzed and confirmed using hematoxylin and eosin-stained slides. Original TMA (from the Euro-FISH program) were centrally prepared using punch needles of 1 mm.\(^{12}\) Eighteen months ago, the four different TMA were stained with 16 different FISH probes (see Table 1) in eight European laboratories (4 probes per laboratory resulting in 2 independent duplicates per probe), analyzed and subsequently stored in the dark at 4°C and normal air pressure. Fifteen TMA, each stained with a different CISH-compatible FISH probe (with the lowest number of lost cores per probe after FISH staining), were chosen for CISH staining. In addition to these slides, one slide cut from TMA n. 3 (kindly provided by the Euro-FISH consortium), stored unstained for the same period of time and under the same conditions as described above, was freshly stained for BCL6-FISH and CISH. Furthermore, a metaphase spread of B-lymphocytes from a healthy donor, used during the Euro-FISH program to validate the BCL6 FISH-probe,\(^{13}\) was re-used for DuoCISH staining. CISH-stained slides were analyzed by bright-field microscopy.

The evaluation involved testing 15 different FISH probes on 11 different WHO lymphoma entities; diffuse large B-cell lymphoma, mantle cell lymphoma, B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, follicular lymphoma, gastric extranodal marginal zone lymphoma (gastric MALT), splenic marginal zone lymphoma, endemic and sporadic Burkitt’s lymphoma, lymphoplasmacytic lymphoma, anaplastic large cell lymphoma, all ALK+, and T-lymphoblastic lymphoma, supplemented with reactive tissues (spleen, tonsil, lymph node and thymus). All entities were evenly distributed over the TMA.

Probes and fluorescence and chromogenic in situ hybridization procedures

Eighteen months ago, four TMA were each stained with 16 different split-signal FISH probes The cut-off of the FISH probes is set at 85%, as determined by counting studies for CE-marking using FISH probes (unpublished data). Fifteen of these TMA (all FISH-stained with different probes) were subsequently DuoCISH-stained (Dako DuoCISH Kit, code no. SK108 and Dako Hematoxylin code S3301, Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark) and analyzed using bright field microscopy.

Four different TMA were used, each containing three samples of endemic Burkitt’s lymphoma, three anaplastic large cell lymphomas, three B-cell chronic lymphocytic leukemia/small lymphocytic lymphomas, four diffuse large B-cell lymphomas, three follicular lymphomas, three lymphoplasmacytic lymphoma, three

Table 1. Tissue microarrays used for DuoCISH that were previously split-signal FISH-stained during the Euro-FISH project.

<table>
<thead>
<tr>
<th>FISH probe</th>
<th>TMA1</th>
<th>TMA2</th>
<th>TMA3</th>
<th>TMA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX5</td>
<td>ALK</td>
<td>IGH</td>
<td>BCL10</td>
<td></td>
</tr>
<tr>
<td>TCRAD</td>
<td>TCRG</td>
<td>TCRB</td>
<td>BCL2</td>
<td></td>
</tr>
<tr>
<td>TCL1</td>
<td>CCND1</td>
<td>MYC</td>
<td>IGL</td>
<td></td>
</tr>
<tr>
<td>TMA1</td>
<td>ALK</td>
<td>IGH</td>
<td>BCL10</td>
<td></td>
</tr>
<tr>
<td>TMA2</td>
<td>PAX5</td>
<td>TCRAD</td>
<td>TMA2</td>
<td></td>
</tr>
<tr>
<td>TMA3</td>
<td>ALK</td>
<td>IGH</td>
<td>TMA3</td>
<td></td>
</tr>
<tr>
<td>TMA4</td>
<td>PAX5</td>
<td>TCRAD</td>
<td>TMA4</td>
<td></td>
</tr>
</tbody>
</table>

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gastrectomy MALT lymphomas, three mantle cell lymphomas, three splenic marginal zone lymphomas, three sporadic Burkitt’s lymphoma, three T-lymphoblastic lymphomas and two reactive cases. The TMA were constructed using each tumor biopsy just once.

Slides were manually stained according to the manufacturer’s manual. A microwave step (Whirlpool JT356, 6th Sense Steam function, 10 min) was used instead of a water bath in the pretreatment procedure (FISH procedure). The hybridizer, 240V, CE-IVD S2451 was used for the pepsin digestion step, denaturation and hybridization step, red and blue chromogenic incubation steps and the counter stain step (FISH and CISH procedures). All previously split-signal FISH-stained slides had been stored in a dark room at 4°C (normal air pressure). One slide, stored unstained at 4°C, was freshly BCL6-stained using the FISH protocol previously described10 and subsequently DuoCISH-stained.

The following FISH probes (with chromosomal localization), serving as templates for DuoCISH-staining, were used: BCL10(1p22); IGK(2p11); ALK(2p23); BCL6(3q27); TCRG(7p14); TCRB(7q34); MYC(8q24); PAX5(9p13); CCND1(11q13); TCRAD(14q11); TCL1(14q32); IGH(14q32); MALT1(18q21); BCL2(18q21); BCL3(19q13); and IGL (22q11).

Data collection

The TMA signals were scored manually according to the manufacturers’ guidelines. A signal was considered co-localized (normal gene segment) if the red and blue signals (red and green for FISH) co-localized or if one red signal and one blue signal were separated by a distance equal to or less than two times the diameter of one signal or if two signals of the same size and color were separated by a distance equal to or less than two times the diameter of one signal. The signals were scored as a split signal (chromosomal break) if two signals of different color were separated by a distance more than two times the diameter of one signal (http://pri.dako.com/split-signal_flyer_interpretation_guide_20308.pdf). Tissue slides were analyzed by microscopic detection without the aid of software. Furthermore, data concerning morphology (good, intermediate, poor/failure), hematoxylin staining intensity (good, acceptable, weak, very weak, failure), signal intensity (strong, moderate, weak, very weak, absent/failure) and actual score (normal = PP, or abnormal = PPP/PB/PB/BR/PPR/PPB or any other combination except PP or no score; with P = purple, B = blue and R = red) were collected using a routine bright field microscope.

Results

During the EuroFISH protocol four TMA, each consisting of 36 cores were prepared with 11 different WHO lymphoma entities, supplemented with reactive benign tissues. Out of the 64 TMA that were previously FISH stained, 15 (each stained with a different FISH probe, see Table 1) were selected for DuoCISH staining, resulting in 540 CISH-stained cores.

After DuoCISH staining cores were analyzed by bright field microscopy and scored for morphology, hematoxylin staining intensity and signal intensity and signal (split or no split). Analysis of these slides was performed in a blinded fashion without prior knowledge of the original split-signal FISH scores or the diagnosis.

A total of 540 cores were DuoCISH stained of which 105 could not be scored; half of these were on three TMA (TMA n.2 stained with CCND1, 17 cases; TMA n. 2 stained with MALT, 19 cases and TMA n.3 stained with MYC, 15 cases). On the other hand, 11 cores that could not be scored in the Euro-FISH protocol were evaluable using CISH. Cores lost during the FISH protocol or lost during the DuoCISH procedure were not used for further analysis. Morphology, hematoxylin staining intensity, signal intensity and diagnostic result after CISH staining were analyzed per tumor entity. These data, except for diagnostic results, are shown in Figure 1. The number of cores of a specific entity with a specific score is represented as a percentage of the total number of cores of that entity.
entity. Although slight differences were seen for morphology (panel A), background staining (panel B) and signal intensity (panel C), overall there were no relevant differences between entities.

To compare actual FISH and CISH scores of split signals, only cores with readable signals were included, resulting in 310 cores. All cores that, during FISH or DuoCISH, were lost or received a designation of failure with respect to the actual score were not used, resulting in eight cores (2.6%) that were found to be differentially scored, meaning that CISH data were inconsistent with the original FISH-data for that particular core. However, these CISH scores were consistent with the Euro-FISH-program independent duplicate TMA (same TMA, different serial coupe, stained and scored by a second laboratory), suggesting that the interpretation of CISH is slightly better than FISH in these cases. Two cores (0.7%), however, were inconsistent with respect to both Euro-FISH duplicates, resulting in overall consistency for 300 cores (96.8%). Figure 2 shows a representative example of a mantle cell lymphoma stained with a CCND1 split-signal probe after split-signal FISH and DuoCISH.

Following DuoCISH staining, morphology was scored and analyzed. Table 2 shows the overall numbers and percentages after FISH staining and, for the same TMA, after DuoCISH staining. As could be expected, due to handling and storage, the overall morphology weakened. DuoCISH staining directly following BCL6-FISH staining, of an unstained Euro-FISH TMA slide (cut, put on a glass slide and stored for 1.5 years at 4°C), resulted in better overall morphology: 24 cores (66.7%) out of 36 were scored as “good”, 8 (22.2%) were scored as “intermediate”, 3 (8.3%) as “poor/failure” and 1 (2.8) as “lost”. The BCL6 FISH probe was also used to test DuoCISH staining on metaphase spreads made from B-lymphocytes of healthy donors. Five metaphases were analyzed showing that the probe localized to chromosome 3q27 (data not shown).

While analyzing the hematoxylin counter-stain of the DuoCISH-stained slides it emerged that the intensity of this counter-stain is associated with the actual signal intensity (Table 3). We, therefore, combined these two datasets and showed that the intensity of the hematoxylin signal corresponds to the signal strength. From our data we conclude that strong hematoxylin and eosin background staining coincides with a strong signal; moderate staining with a moderate signal; weak staining with a weak signal and a failure of hematoxylin and eosin staining almost always results in a failure of signal intensity (highest percentages per intensity are given and shown in bold).

### Discussion

In this study we show that DuoCISH is a reliable and good alternative to split-signal FISH staining. Comparing the score, split or no split, resulting from the DuoCISH procedure in this study to that in the EuroFISH program (total of 310 cores), there were eight cores (2.6%) that were differentially scored. During the Euro-FISH protocol all TMA were scored in duplicate. TMA stained with the same probe were stained and scored by separate laboratories that had no prior knowledge of the results of the other laboratory. Six of the eight CISH-stained cores with aberrant results consistently received the same score as the duplicate FISH TMA. The results for two cores (0.65%) were, however, inconsistent with those of both EuroFISH duplicates, resulting in an overall consistency in score for 300 cores (96.77%). These results indicate that the interpretation of CISH-stained samples is easier than that of

<table>
<thead>
<tr>
<th>CISH signal intensity</th>
<th>good n (%)</th>
<th>moderate n (%)</th>
<th>H&amp;E background staining intensity</th>
<th>very weak n (%)</th>
<th>failure n (%)</th>
<th>lost n (%)</th>
<th>no score n (%)</th>
<th>total, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>No score</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (0.2%)</td>
<td>3</td>
</tr>
<tr>
<td>Failure</td>
<td>1</td>
<td>11</td>
<td>24</td>
<td>54 (10.0%)</td>
<td>36 (6.7%)</td>
<td>0</td>
<td>0</td>
<td>126</td>
</tr>
<tr>
<td>Very weak</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Weak</td>
<td>0</td>
<td>23</td>
<td>60 (11.1%)</td>
<td>38</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>122</td>
</tr>
<tr>
<td>Moderate</td>
<td>16</td>
<td>94 (17.4%)</td>
<td>50</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>168</td>
</tr>
<tr>
<td>Strong</td>
<td>29 (5.4%)</td>
<td>37</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>39 (7.2%)</td>
<td>75</td>
</tr>
<tr>
<td>Lost</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>39 (7.2%)</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>166</td>
<td>145</td>
<td>105</td>
<td>39</td>
<td>1</td>
<td>0</td>
<td>540</td>
</tr>
</tbody>
</table>

Table 3. DuoCISH signal intensity compared to the intensity of the hematoxylin and eosin (H&E) background staining. Highest number of CISH signal intensity and corresponding background staining is given in bold. The percentage of the total number of screened cases is also calculated for these specific combinations.
FISH-stained ones, a finding that fits with the subjective clinical impression of the people who evaluated the samples.

The results of this study show that the overall morphology weakened after prolonged storage of split-signal FISH-stained slides at 4°C. Although only one TMA with 36 cores was DuoCISH-stained directly following FISH, we feel that, from a morphological point of view, it is better to store the slides unstained. On the other hand if morphology scores of “good” and “intermediate” (which might be due to interpersonal variation in interpretation) were considered together, the morphology scoring was only 10% better during the Euro-FISH program.

It could, however, be argued that using old slides rather than fresh re-cuts from the TMA blocks unnecessarily complicated the study and that it decreased the number of usable cores. It should, however, be kept in mind that the availability of some tumor material is limited and, in some cases, can be precious to use just to validate a technique. We did, however, show that the freshly BCL6 FISH-stained TMA performed better after CISH than the previously FISH-stained slides. Nevertheless, we think that re-staining of “old” FISH-stained slides might, in certain cases (referral or second opinion), be desirable.

Additionally we showed that although differences in morphology, hematoxylin staining and CISH signal intensity varied between the tumor entities, none of the entities was easier or more difficult to score. During EuroFISH it appeared that endemic Burkitt’s lymphoma, anaplastic large cell lymphoma, lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, T-lymphoblastic lymphoma and reactive lesions resulted in approximately 88% reliable scores and B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma and sporadic Burkitt’s lymphoma in 90% reliable scores, with the gastric MALT entity being the most difficult to score. Considering only the moderate and good CISH signal intensities, B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, reactive lesions, diffuse large B-cell lymphoma and lymphoplasmacytic lymphoma tumor samples had the best signal intensities (with B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma giving the strongest and lymphoplasmacytic lymphoma the weakest signal) and anaplastic large cell lymphoma, follicular lymphoma, endemic Burkitt’s lymphoma and MALT lymphoma were the four weakest stained samples (with anaplastic large cell lymphoma the weakest and MALT the strongest). We, therefore, conclude that although FISH and CISH signals are generally comparable, a difficult-to-read split-signal FISH does not necessarily result in a weak CISH signal. Furthermore, it is

Figure 2. Mantle cell lymphoma tissue (1 core of a TMA with 36 cores) stained with (A) a CCND1 split-signal probe during the Euro-FISH program (FITC- and Texas-Red labeled) and 1.5 year later (B) with the DuoCISH procedure (blue and red chromogen signals). (A) Leica microscope DM4000B, magnification 400x, colors corrected and clipping of the image after acquisition with Adobe Photoshop. (B) Zeiss Axioskop 2 plus microscope, magnification 400x, colors corrected and clipping of the image after acquisition with Adobe Photoshop.
very relevant that hematoxylin background staining intensity correlates with signal intensity, because it makes routine application of the procedure less dependent on staining variables.

In conclusion, we show, importantly for routine application, that split-signal DuoCISH is at least as reliable as split-signal FISH, a well-documented method for detecting chromosomal breaks in lymphoma samples. Since CISH can be performed in all pathology laboratories on routine samples, our findings suggest that this method could facilitate the classification of lymphomas.

Authorship and Disclosures

AR was the principal investigator and takes primary responsibility for the paper. PC performed the laboratory work for this study and TSP designed the probes. SHD, JC, JCC, CCB, LL, AM, DM, EP and JH contributed the tissue blocks. AR performed the analysis and coordinated the research. AR and JK wrote the paper. None of the authors, except for TSP, who was directly employed by Dako A/S Denmark at the time of this study, reported potential conflicts of interest.

References