Original Articles

Combination of Lamin Immunocytochemistry and In Situ Hybridization for the Analysis of Chromosome Copy Numbers in Tumor Cell Areas With High Nuclear Density

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We describe the application of lamin immunocytochemistry (ICC) and single- or double-target fluorescence in situ hybridization (FISH) on 4 μm thick frozen tissue sections as a method to facilitate scoring of aberrant chromosome copy numbers in colonic tumors. Analysis of FISH signals in colon tissue sections is often hampered by overlap and truncation of epithelial nuclei, due to the density of the epithelial cells. Furthermore, on the basis of nuclear staining it is often difficult to determine whether or not nuclei are overlapping, or adjoining. Therefore, reliable evaluation of (F)ISH signals to screen for genomic changes was until now mainly restricted to isolated nuclei obtained from relatively thick tissue sections.

In this study the applicability of lamin ICC, to stain the nuclear periphery and to distinguish individual nuclei, combined with the FISH procedure is explored to solve this problem for colon epithelium. For ICC we applied the alkaline phosphatase (APase)-Fast Red detection method, since the fluorescent precipitate of this reaction resists extensive proteolytic digestion as needed for efficient FISH on tissue sections.

Chromosome copy numbers could easily be determined in 4 μm thick frozen tissue sections by combining lamin ICC and FISH. The ratio of the copy numbers of the chromosomes 7 and 17 could be determined in frozen tissue sections after combined lamin ICC and double-target FISH.

It is concluded that the combination of lamin ICC and FISH improves chromosome copy number analysis and can be used to investigate genomic changes in different tumor compartments in thin frozen tissue sections. © 1996 Wiley-Liss, Inc.

Key terms: Interphase cytogenetics, tissue sections, alkaline phosphatase, colon, lamin B2

The fluorescence in situ hybridization (FISH) technique is applied as a tool to investigate numerical and structural chromosome aberrations in solid tumors, either as single cell suspensions, frozen tissue sections, or paraffin sections. The evaluation of FISH signals in interphase nuclei of thin sections, however, is hampered by truncation of the nuclei and in some tissues, like colon tumors, also by overlapping of nuclei due to the epithelial cell density. In these cases it is often difficult to distinguish between the individual nuclei and thus to determine accurately the number of FISH signals per single nucleus. In case of colon tumors, FISH is therefore more accurate when applied to single cell suspensions isolated from tissue blocks (5). However, for the detection of clonal heterogeneity in epithelial tumors, it is necessary to detect the aberrant cells within the tissue architecture. For this purpose, use of FISH on tissue sections is therefore preferable.

To solve the problem of FISH signal interpretation in truncated nuclei of sections, several approaches are devel-

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oped to determine the real chromosome copy number. One approach uses the Chromosome Index (i.e., total number of FISH signals per number of nuclei counted) (3, 8). This approach enables the detection of monosomic or tetrasomic cells in cases where these populations make up more than 25% of the total cell number. Other investigators have used serial sections for analysis of chromosome copy numbers. Emmerich et al. (4) analyzed only those nuclei that were localized entirely within three subsequent slides. Hopman et al. (7) estimated the number of chromosome targets and chromosomal imbalances by employing different probes. Alers et al. (1) detected chromosomal loss and gain by statistical evaluation of probe spot frequency distributions using the Kolmogorov-Smirnov test. Recently, Thompson et al. (12) applied FISH to 20 μm thick sections followed by analysis using confocal scanning laser microscopy. This approach is, however, limited for use in routine screening analysis.

In this paper we report a novel approach to solve interpretation problems arising with the evaluation of FISH signals in possible overlapping and truncated nuclei in thin frozen tissue sections. ICC staining of the nuclear contour with a lamin antibody was combined with single- and double-target FISH to determine the FISH signals per individual nucleus.

MATERIALS AND METHODS

Tissue Samples

Fresh tissue samples from 4 patients with colon carcinomas were obtained after surgery and snap frozen in liquid nitrogen.

DNA Probes and Labeling Procedures

Centromere specific probes for human chromosomes 7 and 17 were used (13). The DNA probes were labeled with either biotin-11-dUTP (ENZO, New York, NY), digoxigenin-11-dUTP, or fluorescein-12-dUTP (Boehringer, Mannheim, Germany) in a standard nick-translation reaction.

Single Cell Suspensions

In order to obtain reference values for the individual chromosome copy numbers, to be compared to the data obtained in the tissue sections of the same cases, single cell suspensions were prepared from 50 μm thick sections after pepsin digestion (100 μg/ml pepsin in 0.01 N HCl for 15 min at 37°C). FISH was performed with the centromere specific probes for chromosomes 7 and 17 on single cell suspensions as described before (5), and 200 nuclei per chromosome probe were analysed according to criteria described before (7).

Immunocytochemical Staining Procedures

The monoclonal antibody LN43 was used to detect lamin B2 (2) and was kindly provided by Dr. E.B. Lane (Dundee, U.K.). Four micrometer thick frozen tissue sections were mounted on poly-L-lysine coated slides, air-dried, and briefly fixed in methanol for 1 min at −20°C and in acetone three times for 5 s at room temperature. The slides were air-dried and washed twice for 5 min in PBS containing 0.05% Tween 20 (buffer A), followed by incubation for 45 min at 37°C with the undiluted primary antibody culture supernatant. After two washes of 5 min each with buffer A, the antibodies were detected by incubation with alkaline phosphatase conjugated goat anti-mouse IgG (GAM-APase, diluted 1:25 in buffer A; DAKO A/S, Glostrup, Denmark) for 30 min at 37°C. After two washes in buffer A (5 min each), APase activity was detected by the Fast Red precipitation reaction (10, 11). The standard protocol was performed with staining reagent containing 4 ml 0.2 M Tris-HCl buffer, pH 8.5, 5% poly vinyl alcohol (PVA) (MW 40,000; Sigma, St. Louis, MO), 1 mg naphthol-ASMX-phosphate (Sigma) in 250 μl buffer without PVA, and 5 mg fast red TR salt (Sigma) in 750 μl buffer without PVA. The reaction time was optimized for frozen-tissue sections. The reaction was followed under the light microscope and allowed to run for maximally 10 min.

Single- and Double-Target FISH After Immunocytochemistry

After APase-Fast Red immunostaining of the specific cell protein under investigation, the slides were washed twice for 5 min in buffer A, digested with 100 μg/ml pepsin in 0.01 N HCl for 10 min at 37°C, and washed twice for 5 min in 0.01 N HCl. Thereafter the FISH procedure was performed as described before (6).

For single-target FISH we applied, after immunocytochemical detection of lamin B2, biotinylated probes that were detected with AvFITC, BioGAA, and again AvFITC. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

For double-target FISH, FITC-labeled DNA probes were detected by subsequent incubation with mouse anti-FITC (MAFITC, 1:500), FITC-conjugated rabbit anti-mouse IgG (RAM-FITC, 1:80), and FITC-conjugated swine anti-rabbit IgG (SWAR-FITC, 1:80) (all from DAKO). Digoxigenin-labeled probes were detected with subsequent detection steps of mouse anti-digoxin (MADig, 1:2,000) (Sigma), RAM-FITC, and SWAR-FITC. The biotinylated probes were detected by subsequent incubation with amino acids.

**Fig. 1.** A–B: DAPI DNA counter staining in 4 μm thick frozen tissue sections of colon carcinomas without (A) or with lamin ICC (B). C–I: Results of combined lamin ICC and single- or double-target FISH on 4 μm thick frozen tissue sections of colon carcinomas. C: Combined ICC for lamin and FISH with a probe for chromosome 7 in colon epithelium classified as normal. D,E: Combined ICC for lamin and FISH with a probe for chromosome 7 (D) and DAPI DNA counter staining (E) in a tumor area classified as tetrasomic for chromosome 7. The arrow in E shows stabilization of the nucleus as a result of the Fast Red precipitate. F,G: Combined ICC for lamin and FISH with a probe for chromosome 17 (F) and DAPI DNA counter staining (G) in a tumor area classified as monosomic for chromosome 17. H,I: Combined ICC for lamin and double-target FISH with a probe for chromosome 7 (AMCA, blue) and a probe for chromosome 17 (FITC, green) in colon epithelium classified as trisomic for chromosome 7 and monosomic for chromosome 17. A–G, I, ×125; H, ×50.
methyl coumarin acetic acid (AMCA)-conjugated avidin (AvAMCA, 1:100) (Vector; Brunschwig Chemie, Amsterdam, The Netherlands), biotinylated goat anti-avidin (BioGAA, 1:100) (Vector), and again AvAMCA.

**Evaluation of FISH Signals**

The number of FISH signals was counted per lamin positive nucleus in 10 series of 100 nuclei per slide. For double-target FISH without lamin staining the ratio of the FISH signals for chromosome 17 and 7 was determined in 15 areas of about 2,500 μm². Microphotographs were recorded with the Zeiss Axioskop or with the Metasystem Image Pro System (black and white CCD camera). Fast Red in Red, FITC in green, AMCA and DAPI in blue. The images were printed with a Mitsubishi S3410 thermo sublimination printer.

**RESULTS**

**FISH Results in Frozen Tissue Sections Without Lamin Staining**

The tissue pretreatment steps that are needed to guarantee efficient probe hybridization in FISH, result in a considerable overlap between individual nuclei in frozen tissue sections (Fig. 1A). Because of the extent of nuclear overlap, FISH signals in 4 μm thick frozen tissue sections cannot be assigned properly to individual nuclei, making an evaluation of such FISH preparations questionable.

**APase-Fast Red Lamin Reaction**

The APase-Fast Red reaction that was applied for detection of nuclear lamins, resulted in a strongly fluorescent Fast Red precipitate. Lamin detection resulted in staining of the nuclear contours and allowed to distinguish individual nuclei. The optimal reaction time of the APase-Fast Red precipitation for analysis by fluorescence microscopy was 10 min. A longer reaction time resulted in a more dense precipitate (10), which gave rise to problems with the efficiency of subsequent FISH procedures. The intensity of Fast Red fluorescence and the stability of the precipitate were not reduced after application of the FISH procedures. The nuclear contour staining resulted in less swollen nuclei as compared to nuclei not stained for lamins. As shown in Figure 1B the overlap between nuclei in frozen sections immunocytochemically stained for lamin, was much less than in unstained sections (Fig. 1A).

**FISH Results in Frozen Tissue Sections With Lamin Staining**

Apart from the fact that lamin Immunostained nuclei retained their original size also after the FISH procedure, lamin staining with APase-Fast Red revealed also a clear nuclear contour staining and therefore allowed proper distinction of individual nuclei (Fig. 1C,D,F). As a result the number of FISH signals per individual nucleus could now be determined. For example, Figure 1C shows a normal area directly adjacent to the carcinoma, with many nuclei containing two signals for chromosome 7 (indicative for disomy). Figure 1D,E show a tumor area with four signals for chromosome 7 in part of the nuclei (indicative for tetrasomy), while Figure 1F,G show a tumor area with one signal for chromosome 17 in most nuclei (indicative for monosomy). The real chromosone copy number on basis of counting is underestimated as a result of nuclear truncation in the 4 μm thick tissue sections. To determine the reliability of the combined FISH/nuclear contour staining procedure, the following analyses were made.

1. Comparison of FISH results in sections and in suspensions of single nuclei. FISH results on lamin stained 4 μm thick sections were correlated to the estimations of spot numbers for individual chromosomes in suspensions of single nuclei prepared from a 50 μm thick section of the same sample. In the latter, the effect of truncation is largely absent. For this purpose we selected four colon carcinoma samples with an evident monosomy for chromosome 17 (case 1; 42% of the nuclei), disomy for chromosome 17 (case 2; 83% of the nuclei), trisomy for chromosome 7 (case 3; 56% of the nuclei), or tetrasomy for chromosome 7 (case 4; 49% of the nuclei). As expected, the mean number of FISH signals found in the sections with combined lamin staining is lower as compared to the results in the suspensions, due to truncation of nuclei. The results of this comparison are summarized in Table 1. When comparing these results it should be kept in mind that the results of the nuclear suspensions are biased by admixture of nuclei from normal stromal cells. In contrast, the results for the tissue sections were obtained by screening selected tumor areas.

2. Comparison of frequency distributions of FISH signals in sections and in suspensions of single nuclei. The FISH signal frequency distributions of the analysed nuclei in 4 μm thick sections, as well as in suspensions, are compared to each other in Figure 2. From this figure it is
obvious that an underestimation of the real chromosome copy number occurs in the 4 μm thick sections as a result of truncation of the nuclei. These frequency distribution patterns, however, are in accord with earlier observations of ISH studies on 4 to 6 μm thick tissue sections (7, 9). From this figure it becomes particularly obvious that an
apparent down shift of the number of signals is seen when
comparing results from sections to those obtained in the
suspicions of single nuclei.

**Nuclear Lamin ICC With Double-Target FISH**

For combined lamin ICC and double-target FISH (Fig.
1H, I) a chromosome 7: chromosome 17 ratio of 1.0 ± 0.1
was found in areas with disomy for chromosome 7 and
disomy for chromosome 17. In areas with a trisomy for
chromosome 7 and monosomy for chromosome 17 this
ratio was 2.9 ± 0.4. When double-target FISH was per-
formed on these sections without preceding lamin stain-
ing, the average ratios were 1.0 ± 0.1 and 2.9 ± 0.2,
respectively. These are, however, results obtained by
counting the number of FISH signals for the individual
chromosomes in a tumor area. In this latter case no in-
formation is obtained about the number of FISH signals
per individual nucleus.

**DISCUSSION**

ISH on tissue sections is increasingly used to detect
genetic aberrations in solid tumors. However, accurate
analysis of the absolute number of FISH signals is ham-
pered by truncation of the nuclei, although an estimate of
the chromosome copy number can be made on the basis
of hybridization of serial sections, references within the
tissue sections itself, and after mathematical correction
(7, 8, 9). In some (pre)malignancies, such as colonic and
cervical tumors, an additional problem is introduced by
the overlap of the epithelial nuclei, due to the cell den-
sity, which makes it difficult to distinguish the individual
nuclei, even in 4 μm thick sections. This problem is en-
hanced when these samples have undergone the prote-
olytic pretreatment steps needed for the ISH procedure,
resulting in an even fuzzy appearance of the nuclei.
In this paper a novel approach is reported to partly over-
come these problems.

We applied the sensitive APase-Fast Red reaction for
ICC staining of lamin, which allows a combination with
ISH, since the Fast Red precipitate is resistant to the ex-
tensive enzymatic digestion step, necessary for the reduc-
tion of autofluorescence and to allow penetration of DNA
probes and antibodies. Lamin immunostaining with
APase-Fast Red leads to an apparent stabilization of the
nucleus, presumably as a result of physical trapping of the
DNA within an artificial matrix (Fig. 1E, arrow). As a
result the extent of nuclear overlap in the final ISH prep-
paration is drastically decreased. Furthermore, this pro-
tein-Fast Red matrix allows an even more rigorous pro-

telytic pretreatment of the tissue, resulting in a more

efficient ISH reaction. Adjoining nuclei in such lamin
stained preparations, that could not be distinguished with
DAPI-counter staining, could be separately recognized by
the contour staining of lamin, and evaluation of these
individual nuclei was therefore improved. Taken to-
gegether, staining for lamin makes it possible to analyse
tumor sections by ISH that could otherwise not be eval-
uated. Also it allows the analysis of individual nuclei, by
which genome heterogeneity can be detected in tumor
samples.

Although we can now accurately analyse the number of
FISH signals in thin frozen tissue sections of colon carci-
nomas, the results do not always provide the true chro-
mosome copy number, since truncation of nuclei occurs.
By counting several fields of approximately 100 nuclei
we could obtain a reasonable estimate of the true copy
number for individual chromosomes. We can thus detect
chromosome aberrations in tissue sections, although the
detected deviations are always less prominent as com-
pared to the results obtained in suspensions of single nu-
clei. The results are always an underestimation of the real
chromosome copy numbers. The extent of deviation from
the real copy number decreases with the thickness of
the tissue section, but as a result nuclear overlap will
increase. To obtain a balance between these two phe-
nomena we chose to use 4 μm thick sections in the case
of colon carcinomas. It might well be that for the analysis
of other tumor types thicker sections can be used. To
determine the absolute chromosome copy number, even
thicker sections should be analysed using confocal laser
scanning microscopy (12).

In another approach to determine chromosome imbal-
ances in colon tumor tissue sections, we performed dou-
ble-target FISH with and without lamin staining. In dou-
ble-target FISH without lamin staining, only an average
chromosome copy ratio after evaluation of areas of (over-
lapping) nuclei can be determined, but not the chromosomal
ploidy. To obtain more precise information about the
chromosomal content per nucleus, combined lamin stain-
ing with double-target FISH is therefore preferable. What
remains to be incorporated into this approach is the phe-
notyping of the aberrant cell type, e.g., by means of dif-
ferentiation markers. Compared with other methods used
for the detection of chromosomal aberrations in sections
(3, 4, 7, 8), the procedure described in this paper has the
major advantage that in one section the chromosome
copy number, as well as chromosome imbalances, can be
detected.

We conclude that combined ICC detection of nuclear
lamin and single- or double-target FISH allow a more ac-
curate estimation of chromosome copy numbers in thin
frozen tissue sections. The method is of particular help in
(tumor) tissue with densely packed nuclei.

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