BRIEF METHODS

Initial Intermittent Microwave Irradiation for Fluorescence In Situ Hybridization Analysis in Paraffin-Embedded Tissue Sections of Gastrointestinal Neoplasia

Yasuhiko Kitayama, Hisaki Igarashi, and Haruhiko Sugimura

First Department of Pathology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka, Japan

Distinct hybridization signals for formalin-fixed paraffin-embedded tissue sections are difficult to obtain using fluorescence in situ hybridization (FISH), because various conditions, including the fixation procedure, often influence the quality of hybridization. Thus, only a few studies involving the analysis of paraffin-embedded tissue sections using FISH have been reported. In particular, gastrointestinal tumor specimens are notoriously difficult to handle using standard FISH and conventional cytogenetic techniques because of frequent necrosis, contamination with inflammatory and normal stromal cells, and the poor attachment of the tumor cells (Kitayama et al, 1995). Present techniques involving the use of microwave ovens for antigen retrieval (Igarashi et al, 1994; Taylor et al, 1994) in formalin-fixed, paraffin-embedded tissues have led to the suggestion that FISH signals might be enhanced if an appropriate heat treatment is included during sample preparation. To avoid the above-mentioned problems associated with paraffin sections, the FISH protocol was thus modified to incorporate an intermittent, short-term microwave (MW) treatment during the initial period of hybridization. This report describes the first application of MW-intermittent irradiation to formaldehyde-fixed paraffin-embedded tissue sections for FISH analysis. The FISH signals obtained using this modified method and digoxigenin-labeled probes showed a significant improvement compared with those obtained using the standard FISH method (Pinkel et al, 1986). This new technique thus enables the analysis of various paraffin-embedded tissue sections with poor or uncontrollable fixation conditions.

Samples of gastric tumors were obtained from resected specimens submitted to the Department of Pathology, Hamamatsu University School of Medicine, (Hamamatsu, Japan), for diagnosis. The tissue

samples had been fixed in a 4% neutral buffer of formaldehyde for various terms at room temperature, routinely processed with paraffin wax, and stored for up to 5 years at room temperature. Formalin-fixed paraffin-embedded tissue sections (6 μ m thick) of noncancerous gastric mucosa, carcinomas, and normal lymph nodes that had been processed using three different fixation terms (1 day, 2 weeks, and 4 weeks or more), were also analyzed using the modified FISH technique. A panel of 18 centromeric α -satellite DNA probes and locus specific identifier probes (c-myc and p53) were purchased from Vysis Inc. (Downers Grove, Illinois). All of the probes had been labeled with either orange (Cy 3) or green (FITC) using digoxigenin-11-dUTP and nick translation. The sections were deparaffinized by five successive, 3 minute washes in xylene followed by five washes in ethanol and MW-heating for 10 minutes in a 0.01 M citrate buffer (pH 6.0). After treatment in 0.2% pepsin/0.01 N HCl for 10 minutes at 37° C, the samples were aged in 0.1% NP-40/2 $\times$ SSC for 10 minutes at 37° C and their DNA was denatured by treatment in 70% formamide/2 $\times$ SCC for 5 minutes at 85° C. Ten microliters of the probe solution (hybridization buffer, 7 μ l; probe, 1 μ l; DW, 2 μ l) was then placed on a glass slide with a cover slip. The sample slides with the hybridization mixture were then put in a MW processor (MI-77, Azumaya Company, Tokyo, Japan). The samples were irradiated for 3 seconds at 2 second intervals (2.45 GHz, 300 W) with a temperature sensor set at 42° C, as described previously (Kitayama et al 1999). Each specimen was subjected to MW-intermittent irradiation during the initial period of in situ hybridization (ISH) for either 15 minutes or 1 hour. DAPI II (4,6-diamidini-2-phenylindol, 125 ng/ml, Vysis Inc.) was used for nuclear counterstaining. The samples were promptly observed using a fluorescence microscope equipped with epifluorescence filters and a photometric CCD camera (Quantix 1400, Olympus Optical Company, Ltd., Tokyo, Japan). The captured images were digitized and stored in an image analysis program (Cyto-Vision; Applied Imaging, Olympus). The number of signals per cell was counted for a total of 100–200 cell

Received January 27, 2000.

This work is supported by the Smoking Research Foundation.

Address reprint requests to: Dr. H. Sugimura, First Department of Pathology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31 Japan. Fax: 053 435 2225.

nuclei, and the signal intensities for the different periods of hybridization were simultaneously compared. The prevalence of lymphocytes exhibiting an unambiguous diploid signal was monitored for the three different fixation periods according to the period of hybridization (mean $\pm$ SD).

As shown in Figure 1, A and B, 93% of the normal lymphocytes that had been fixed for 2 weeks and subjected to 1 hour of MW-intermittent irradiation at the beginning of hybridization yielded normal diploid signals after an additional 3 hours of hybridization, whereas only 58% of the normal lymphocytes treated with MW-intermittent irradiation for 15 minutes yielded

Figure 1 (A)

Figure 1.

Average percentage of the cells (mean $\pm$ SD) with a 2-spot signal for normal lymphocytes in samples hybridized with 20 probes and subjected to 15 minutes (A) and 1 hour (B) of microwave intermittent irradiation at the beginning of in situ hybridization (ISH). The tissue sections had been fixed in formalin for three different terms ($\circ$ $\bullet$ fixed for 1 day; $\triangle$ $\blacktriangle$ fixed for 2 weeks, and $\square$ $\blacksquare$ fixed for more than 4 weeks). A significant difference in signal intensity was observed in samples that were fixed for 2 weeks and then subjected to intermittent microwave (MW) irradiation for 1 hour versus 15 minutes (total ISH time = 4 hours; mean $\pm$ SD = 93 ± 2.3 vs 58 ± 7.9 , $p < 0.001$).

signals (mean $\pm$ SD = 93 ± 2.3 vs 58 ± 7.9 , $p < 0.001$). All of the probes that were tested produced a distinct 2-spot signals with no variations in staining intensity in 94% of the normal cell nuclei of samples fixed for 1 day and subjected to 1 hour of MW-intermittent irradiation and an additional 6 hours of hybridization. Distinct signals were also obtained in paraffin sections of normal gastric mucosa that had been fixed for 2 weeks and treated with MW-intermittent irradiation for 1 hour followed by an additional 6 hours of incubation (Fig. 2A). By subjecting the samples to 1 hour of MW-intermittent irradiation, even signals from cancerous tissues that had been fixed for 4 weeks or more could be clearly detected (Fig. 2B). The MW irradiation protocol was designed with several characteristics of microwaves in mind. A previous report had shown that MW-intermittent irradiation treated samples reach a maximum hybridization threshold after 30 minutes, and that the signals from fresh sections gradually start to diminish after 6 hours of irradiation. The reason for this signal deterioration might be that the binding of the fluorescent probes is disrupted by excessive microwave vibrations. Intermittent microwave irradiation

Figure 2.

Chromosome 16 centromere in paraffin sections of gastric mucosa fixed for 2 weeks (green, A) and sex chromosome X centromere in paraffin sections of gastric mucosa with signet ring cell carcinoma fixed for more than 4 weeks (green, B) after 6 hours of ISH with 1 hour of microwave intermittent irradiation at the beginning of the hybridization period. Distinct 2-spots signals were found in epithelial and stromal cell nuclei, and less numbers of signals (1 or 0) in cancer cells.

seems to be beneficial in terms of maintaining a constant temperature and in preventing the slides from drying out (Kitayama et al, 1999). However, simple intermittent MW treatment is not ideal for obtaining good hybridization signals, and long-term intermittent MW irradiation may be harmful, as described above. An MW-intermittent irradiation treatment of either 15 minutes or 1 hour at the beginning of hybridization was therefore adopted. None of the negative controls, excluding the 0.2% pepsin pretreatment, MW heating and MW-intermittent irradiation, generated any distinct or constant signals in the paraffin tissue sections (data not shown). This protocol might also minimize background noise. Although the reason for the improvement in sensitivity is not clearly understood, the simultaneous enhancement of intermolecular movement and binding efficiency as a result of MW irradiation seems to be a likely mechanism (Lan et al, 1996; Sibony et al, 1995). In conclusion, the modified FISH technique involving MW processing described in this report should allow reproducible and unambiguous information on chromosomal instabilities to be obtained from formalin-fixed paraffin-embedded tissue sections of various solid tumors in humans. The described protocol, which includes an initial intermittent microwave treatment, is recommended as a simple, powerful, and highly reproducible technique for examining paraffin-embedded archives using FISH.

References

- Igarashi H, Sugimura H, Maruyama K, Kitayama Y, Ohta I, Suzuki M, Tanaka M, Dobashi Y, and Kino I (1994). Alteration of immunoreactivity by hydrated autoclaving, microwave treatment, and simple heating of paraffin-embedded tissue sections. *APMIS* 102:295–307.
- Kitayama Y, Igarashi H, and Sugimura H (1999). Amplification of FISH signals using intermittent microwave irradiation for analysis of chromosomal instability in gastric cancer. *J Clin Pathol* 52:357–359.
- Kiatyama Y, Nakamura S, Sugimura H, and Kino I (1995). Cytophotometric and flow cytometric DNA content of isolated glands in gastric neoplasia. *Gut* 36:516–521.
- Lan HY, Mu W, NG YY, Nikolic-paterson DJ, and Atkins RC (1996). A simple, reliable, and sensitive method for nonradioactive in situ hybridization; use of microwave heating to improve hybridization efficiency and preserve tissue morphology. *J Histochem Cytochem* 44:281–287.
- Pinkel D, Straume T, and Gray JW (1986). Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc Natl Acad Sci USA* 83:2934–2938.
- Sibony M, Commo F, Callard P, and Gasc JM (1995). Enhancement of mRNA in situ hybridization signal by microwave heating. *Lab Invest* 73:586–591.
- Taylor CR, Shi SR, Chaiwun B, Young L, Imam SA, and Cote RJ (1994). Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. *Human Pathol* 25:263–270.