Retrieval of proliferating cell nuclear antigen (PCNA) immunoreactivity on formalin-fixed tissues—comparison of effects of microwave irradiation and autoclave processing—

Junko SAKIYAMA, Mitsuko ICHIMURA, Hiroko TOHGE, Hiroshi ENDO, Kaori KAWAKAMI and Yuki KAWATANI

Abstract

Using paraffin-embedded tissue sections of liver cancer obtained from autopsy which had been preserved in 10% buffered formalin solution for 6 months while PCNA immunoreactivity was lost, we examined the effects of heat processing by either microwave (MW) and autoclave (AC) in the presence of various processing solution. It appeared that AC processing took shorter time period than MW irradiation to restore equal immunoreactivity. With regard to immunoreactivity retrieval by MW irradiation, however, variation of the degree of retrieval depending on processing time was smaller than in AC, and so the stable consequences were obtained. Although AC processed tissues tended to be stained deep, prolonged processing time presented strong background staining and blurred nuclear margins which made it difficult to estimate the positive cell count. As for the effects of processing solution, there was little difference in retrieval of PCNA among 0.01 M citrate buffer (pH 6.0), saturated solution of lead thiocyanate and distilled water, but the least background staining was observed with distilled water. These observations suggest that MW irradiation of which effect of retrieval is less dependent of processing time and with the least background stainability, is superior to AC processing for PCNA immunoreactivity retrieval on formalin-fixed tissues.

Key words: PCNA, microwave, autoclave, immunohistological staining, formalin fixation

Introduction

In various tumors, markers of cell growth kinetics become important indexes for histological grade of malignancy, therapy response and prognosis prediction. Thus, demonstration of proliferating cell nuclear antigen (PCNA) has recently been indicated to be a target for marking of proliferative activity in tissue sections. It is non-histone type protein with the molecular weight of 36 kD and shown to be the same protein as supporting factor of DNA polymerase δ which appears in cell nuclei of G1, S and G2 phases of cell cycle. The monoclonal antibody PC10 against PCNA was developed in 1990 by Hall et al. and has quickly been wide-spread because of its applicability to formalin-fixed paraffin-embedded specimen. It was reported that the immunoreactivity was markedly decreased after 24 hours of fixation in formalin. For many years it has been a
usual method for immunocytochemistry to expose antigenic site using enzymatic digestion, e.g., with trypsin, but recently it was reported that microwave (MW) irradiation was an excellent method for antigen retrieval at least in some cellular antigens⁷⁻⁹. Using this method we could enhance PCNA staining in tissue sections after 24 hours of fixation in formalin. Another method for immunoreactivity retrieval is autoclave (AC) processing. But the mechanism of PCNA immunoreactivity retrieval by MW irradiation or AC processing is not clearly understood. In this study we made a comparison on the effects of PCNA retrieval between MW irradiation and autoclaving under various conditions including temperature, period of process and type of processing solutions.

**Materials and methods**

1. **Tissues**
   
   Autopsy material of liver cancer preserved in 10% buffered formalin for 6 months was used for the study. Four μm serial sections were made after paraffin embedding with usual method. Sections were mounted on the 3-aminopropyl-triethoxysilane (APES)-coated slides, and dried perfectly overnight in the incubator at 37°C to prevent detachment during heat processing by MW or AC.

2. **Retrieval method of PCNA immunoreactivity**
   
   a) **Apparatus**
   
   - Microwave quick preparation fixer MF-2 (Nisshin EM Co., Ltd.)
   - Autoclave ASU-3022 (Sakura Seiki Co., Ltd.)
   
   b) **Processing solution**
   
   - 0.01M citrate buffer, pH6.0
   - Saturated solution of lead thiocyanate
   - Distilled water
   
   c) **Processing**

   After dewaxing, slides were washed with distilled water, put into the thermostable container with processing solution, and then this container was set in a bigger container filled with water to prevent boiling. Continuous MW irradiation for 5min (1cycle) was repeated, namely for 15min (3cycles), 30min (6cycles) or 45min (9cycles). AC processing was also applied at 121°C or 100°C, for 15 or 30min, respectively.

3. **Immunohistological staining**
   
   a) **Antibodies**
   
   - Primary antibody: monoclonal mouse anti-proliferating cell nuclear antigen (DAKO-PCNA, PC10)
   - Negative control: mouse IgG2a negative control (DAKO)
   - VECTASTAIN ABC-PO (mouse IgG) kit (Funakoshi Co., Ltd.)

   b) **Staining**

   After MW irradiation or AC processing the sections were allowed to cool to room temperature, then they were washed with 0.01M phosphate buffer (PBS, pH7.8). Endogenous peroxidases activity was blocked with methanol containing 3% hydrogen peroxidases at room temperature for 30min and washed with PBS, and non-specific binding was blocked by the incubation with 10% normal horse serum at room temperature for 30min. Sections were hereafter incubated overnight at 4°C with the primary antibody PC10 in a 1 : 500 dilution with PBS, then washed with PBS, and applied ABC method by VECTASTAIN ABC kit according to the instructions. The color was developed using DAB-cobalt method for 5min. Sections were counterstained with methyl green. Incubation of sections with omission of the primary antibody was performed as negative control, as well as incubation of sections with an immunoglobulin subclass-matched unrelated primary
monoclonal antibody. Control sections had no positive stains of PCNA.

4. Evaluation

The corresponding regions of serial sections were observed and PCNA positive cell nuclei per 1,000 cancer cells were counted with oil immersion.

**Results**

The specimens which had usual immunostaining without antigen retrieval processing were defined as non-processed. The apparent retrieval of PCNA immunoreactivity in MW irradiation and AC processing were compared with non-processed (Fig. 1). Numbers of PCNA-positive nuclei in each processing condition is also shown on Table 1. In MW irradiations, the PCNA positive numbers of cancer cells on 30min processing were larger than those of 15min, but no difference was seen between 30min and 45min. On the other hand, 15min AC processing at 121°C showed larger PCNA positive numbers of cancer cells than 30min. Moreover, the PCNA positive numbers at 100°C were obviously low compared with those at 121°C. Little difference of PCNA retrieval was seen among the three kinds of retrieval processing solution both in MW irradiation and AC processing (Table 1).

Regarding the 15-min processing time, PCNA was stained deeper after AC processing at 121°C than MW irradiation (Fig. 2, a&b). But AC processing at 121°C for 30min (Fig. 2, d)
showed ① distinct background staining (cytoplasm in particular), ② disruptive change in tissue structure, ③ blurred nuclear margins in PCNA positive nuclei. These findings ① and ② were seen in all retrieval processing solution and finding ③ was more remarkable in citrate buffer. In these reasons, AC processing at 121℃ for 30min was apt to become difficult to distinguish between positive and negative results in comparison with those for 15min. On the other hand, in 30min MW irradiation, the positive characters of PCNA staining were clearer than in 15min and easier to distinguish positive cells from negative. Even in 45min MW irradiation, there were no disadvantages such as strongly stained background, destruction of tissue or blurred phenomenon which were seen in AC processing for 30min. These stable results were obtained with all retrieval processing solutions.

Discussion
Shi et al. reported that the best results were provided when saturated solution of lead thiocyanate or 1% zinc sulfate were used as
processing solution for MW retrieval, although they did not examine on PCNA staining. Suurmeijer et al.\textsuperscript{10} found the similar results with 4% aluminum colloidal solution. They suggested that metal salts in the processing solution were favorable for antigen retrieval. On the other hand, Catoretti et al.\textsuperscript{11} reported that 0.01M citrate buffer (pH 6.0) was satisfactory in many respects such as antigen retrieval and low background staining. Igarashi et al.\textsuperscript{12} also recognized the significant enhancement of PCNA immunoreactivity in tissues fixed with formalin for a week when the following processing solutions were employed; distilled water at 60°C overnight or zinc sulfate at 90°C for 10min, but they didn’t think that zinc sulfate brought more efficient retrieval than distilled water. Haerslev et al.\textsuperscript{13} obtained the satisfactory results with distilled water using tissues such as breast cancer processed by MW, but they had less satisfactory results with citrate buffer. In the present study, we examined efficiencies of three processing solutions (citrate buffer, lead thiocyanate and distilled water) for PCNA retrieval. Although each solution showed no significant difference for the number of PCNA-positive cancer cells, processing with distilled water resulted in lowest background staining. Haerslev et al.\textsuperscript{13} reported that PCNA staining was also satisfactory when MW irradiation was performed above the boiling point for 25min regardless of fixation periods. Malmström et al.\textsuperscript{14} studied the relationship between histological grade of malignancy and PCNA staining in urinary bladder cancer. They suggested that a positive correlation was found between extension and intensity of PCNA immunoreactivity retrieval by MW irradiation and histological grade. They also reported that an increase in the heating time resulted in cytoplasmic staining in more recent specimens (less than two weeks fixation), while no increase was found in older ones despite heating for up to 30min. In the present study, all MW irradiational conditions or AC processing at 121°C for 15min showed comparatively stable results in all processing solutions, but the findings such as dark background and destruction of tissue structure were seen in the specimens with AC processing at 121°C for 30min. In particular, such findings were found in processing solutions except for an distilled water. Prolonged MW irradiation brought the satisfactory results compared with the previous studies mentioned above, possibly due to long fixation time of our materials. Although AC processing at 121°C is more convenient to perform than MW irradiation, MW irradiation brings better results in other aspects such as morphological preservation and easy distinction between negative and positive cells. In conclusion, MW irradiation is thought to be superior to AC processing for PCNA immunoreactivity retrieval on formalin-fixed tissues, and distilled water as processing solution is enough to get the satisfactory results. Further studies on the mechanisms of immunoreactivity retrieval by MW irradiation are needed to examine on tissues fixed for various time periods.

References


長期ホルマリン固定により失活した Proliferating Cell Nuclear Antigen（PCNA）の免疫反応性回復条件の基礎的検討
—マイクロウェーブ、オートクレーブの影響について—

崎山順子 一村光子 唐下博子 遠藤 浩 川上香利1) 河谷友紀2)

要約
10%緩衝ホルマリンに6ヶ月間浸漬していた剖検材料（肝臓癌）のパラフィン包埋後の組織切片を用いて、ホルマリンの固定作用により失活した PCNA の免疫反応性の回復にマイクロウェーブ（MW）及びオートクレーブ（AC）による熱処理とその時用いる処理溶液が、どのような影響を与えるかについて検討した。その結果、同等の免疫反応性を回復するのには、AC 処理の方が MW 照射より短時間でよいことが解った。しかし、MW 照射による免疫反応性回復では、処理時間による影響が AC 処理に比べて少なく、安定した結果が得られた。また、PCNA の染色所見については、AC 処理の方が濃く染まる傾向が見られた。しかし、処理時間が長くなるとパックグラウンドの染色性が高くなる、核の周囲にじみ現象が見られる等の所見があり判定に困難をきたした。一方、処理溶液についてはクエン酸（0.01M pH6.0）、チオシアン酸鉛鉱和溶液、蒸留水について検討を行った結果、PCNA の回復には差は認められなかったが、パックグラウンドの染色については蒸留水が最も少なかった。以上の結果より、ホルマリン固定により失活した PCNA の免疫反応性を回復するには、MW 照射の方が AC 処理より処理時間に関係なく安定した染色性が得られ、なおかつパックグラウンドの染色性が少ない等の点で優れていることが示唆された。

キーワード：PCNA、マイクロウェーブ、オートクレーブ、免疫組織染色、ホルマリン固定

岡山大学医療技術短期大学部衛生技術学科
1）鏡島記念病院
2）福山検査センター