Antigen Retrieval of E-cadherin with Citrate Buffer and TWEEN from Formalin-fixed, Paraffin-embedded Tissue by Microwave Oven Heating

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ABSTRACT. We developed a novel method for retrieving E-cadherin antigen from formalin-fixed, paraffin-embedded archival liver tissue sections, and succeeded in clearly revealing the presence of E-cadherin by immunohistochemistry. This method consists of microwave heating of the sections submerged in 0.1 mol/l citrate buffer solution with 0.1% Tween 20. The addition of 0.1% Tween 20 was considered to be crucial for the better retrieval of the antigen. With this treatment, paraffin-embedded liver tissues stored for anywhere from a few months up to 10 years can be utilized and they should still exhibit a strong immunoreactivity with anti-E-cadherin antibody along the cell membrane between hepatocytes. It is hoped that our method will facilitate studies of cadherin biology and its diagnostic and prognostic implications.

Key words: E-cadherin — retrieval — antigenicity — microwave heating — immunohistochemistry

The diagnostic and prognostic implications of E-cadherin immunostaining in human tumors have been widely documented and accepted.[1-9] However, the inherent drawbacks of the epitope and the antibody produced have restricted its application to fresh material.[8] The epitope does not seem to survive after routine fixations such as formaldehyde or alcohol, and only a few trials have been done and been reported to be successful with formalin-fixed materials.[3] In our hands, these procedures did not give good staining results no matter how exactly we followed their procedures, and whatever minor modifications we made in their procedures. After a tremendous number of attempts, we finally succeeded in retrieving E-cadherin in formalin-fixed archival tissue sections, and in revealing it by immunohistochemical techniques. The staining quality of our procedure is excellent and consistent, and it is applicable no matter how old the fixed materials are. Herein, we present this procedure and hope it will facilitate immunohistochemical studies of E-cadherin.

MATERIALS AND METHODS

Specimens

Archival formalin-fixed and paraffin-embedded liver tissues including those of hepatocellular carcinoma were utilized for this study. These tissues had been surgically removed, fixed in neutral buffered formalin (for about 24hrs),

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routinely processed, paraffin-embedded, and stored in the Department of Pathology, Kawasaki Medical School for anywhere from a few months up to 10 years before use. Freshly flozen liver tissue taken surgically was utilized as a control.

Preparation

Four μm-thick paraffin sections were cut, placed on silanized glass slides and deparaffinized in xylene. Then, they were rehydrated in graded alcohols and washed three times in Tris-buffered saline solution with 1 mM calcium (TBS+).

Antigen retrieval

We used a Panasosic model NE-M330 household microwave oven. Preliminary attempts were made with a variety of submerging solutions during microwave oven heating and it was found that the following solutions (Table 1) and durations of exposure were suitable.

TABLE 1. Submerging solutions for antigen retrieval during microwave oven heating

solutions	staining intensity
distilled water	0
PBS	0
TBS+	0
PBS & Tween	1
citrate buffer	1
citrate buffer & Tween	3

A glass beaker containing 800 ml of 0.1 mol/l citrate buffer solution with 0.1% Tween 20 (KATAYAMA CHEMICAL, Osaka, Japan) at final pH 6.9 was preheated for 5 min at the maximum power (700 W) of the microwave oven. Then, up to 20 sections were stacked horizontally in the preheated solytion and were further irradiated at the same power for 5 min to boil them at 100℃. This procedure was repeated three times in total. It is important to note that during microwave heating the sections must be submerged in the fluid all the time. After heating, the glass beaker was removed from the oven and left at room temperature for 15 min to cool down. Sections were then rinsed in TBS+ solution three times for 5 min each. (Fig 1)

1. Deparaffinize sections in xylene.
2. After washing with TBS+ solution, place them in a glass beaker with 0.1 mol/l citrate buffer solution to which 0.1% Tween has been added.
3. Leave the glass beaker in the center of the microwave oven.
4. Heat the solution by 5 min irradiation at maximum power (700 W) three times.
5. Remove it from the oven and let it cool down.
6. Rinse the sections in TBS+ solution three times; 5 min each.

Fig 1. The outline of antigen retrieval using a microwave oven

Immunostaining

We have tried several enzymes for the retrieval of the antigen without microwave treatment. The results we obtained clearly showed that the enzymatic treatment did not affect the results at all (Table 2). We concluded, therefore, that the following immunostaining procedure after microwave treatment was the best.

The avidin-biotin-peroxidase complex (ABC) method was used for immunostaining.[10] To block endogenous peroxidase activity, the specimens were immersed in methanol with 0.3% hydrogen peroxide for 30 min and

TABLE 2. Effects of proteases on antigen retrieval without microwave heating

proteases	staining intensity
Trypsin(30 min.)	0
Trypsin(60 min.)	0
Trypsin(120 min.)	0
Pepsin	0
Pronase E	0
Pepsin/Pronase E	0

rinsed with TBS+. After incubation with 3.0% normal horse serum to block nonspecific binding, the sections were incubated with mouse anti-human E-cadherin monoclonal antibody (HECD-1 : TAKARA, Kyoto, Japan) diluted at 1 : 500 in TBS+ solution with 1.0% (v/v) fetal bovine albumin for 24 hrs at 4℃ in a moist chamber. They were rinsed with TBS+ solution and then reacted with biotinylated horse anti-mouse IgG antibody (VECTASTAIN ABC Kit, Vector, Inc Burlingame, USA) diluted at 1 : 200 in TBS+ solution containing 1.0% normal horse serum for 50 min at room temperature. After washing, the sections were incubated with ABC reagent (VECTASTAIN ABC Kit, Vector, Inc Burlingame, USA) for 50 min at room temperature. Finally, they were reacted in a solution with 40 mg 3,3'-diaminobenzidine tetrahydrochloride in 200 ml of 0.05 mol/l TRIS-HCL buffer (pH 7.6) and 40 μl 30% hydrogen peroxide. Then they were counterstained with Carazzi's hematoxylin, and mounted. (Fig 2)

1. Block endogenous peroxidase activity with 0.3% H_2O_2 for 30 min.
2. Wash with TBS+.
3. Block nonspecific binding of immunoglobulin with 3.0% normal horse serum.
3. React with primary antibody (HECD-1) for 24 hr at 4℃ in a moist chamber.
4. React with biotinylated anti-mouse IgG antibody diluted in TBS+ containing 1.0% normal horse serum for 50 min at room temperature.
5. Wash with TBS+.
6. React with ABC reagent for 50 min at room temperature.
7. Colorize with diaminobenzidine.
8. Counterstain with Carazzi's hematoxylin.

Fig 2. The outline of immunohistochemical staining

Evaluation

The intensity of E-cadherin expression in normal and cancerous liver tissue was examined light microscopically. First, we confirmed that distinct linear immunopositivity was seen at the cell-to-cell junction between hepatocytes as well as cholangiolar epithelia using freshly prepared normal liver tissue. However, it was absent in formalin-fixed, paraffin-embedded tissue sections without microwave treatment. Based on these results, we arbitrarily devised the following scoring system to be used for staining intensities; namely, 3: strong (nearly equal to that of fresh material), 2; moderate, 1; weak, 0; absent (staining intensity nearly equal to formalin-fixed, paraffin-embedded sections without antigen retrieval). This system was applied to the results we obtained in all procedures. Formalin-fixed, paraffin-embedded tissues stored for periods of time ranging from 1 month to 10 years were tested in the same manner. Normal and cancerous liver tissues were compared in their intensity and distribution pattern.

RESULTS

The immunopositivity of E-cadherin using fresh material is shown in Fig 3. Distinct linear brown immunopositivity was seen at the cell-to-cell junction between hepatocytes. The formalin-fixed, paraffin-embedded tissue sections without any antigen retrieval procedures showed complete negativity. After microwave heating with 0.1% Tween 20 in citrate buffer solution, paraffin-embedded liver tissue exhibited a strong immunoreactivity with the anti E-cadherin antibody along the plasma membrane of hepatocytes and cholangiolar epithelia (Fig 4). Hepatocellular carcinoma cells were also stained positively and that result will be reported elsewhere.

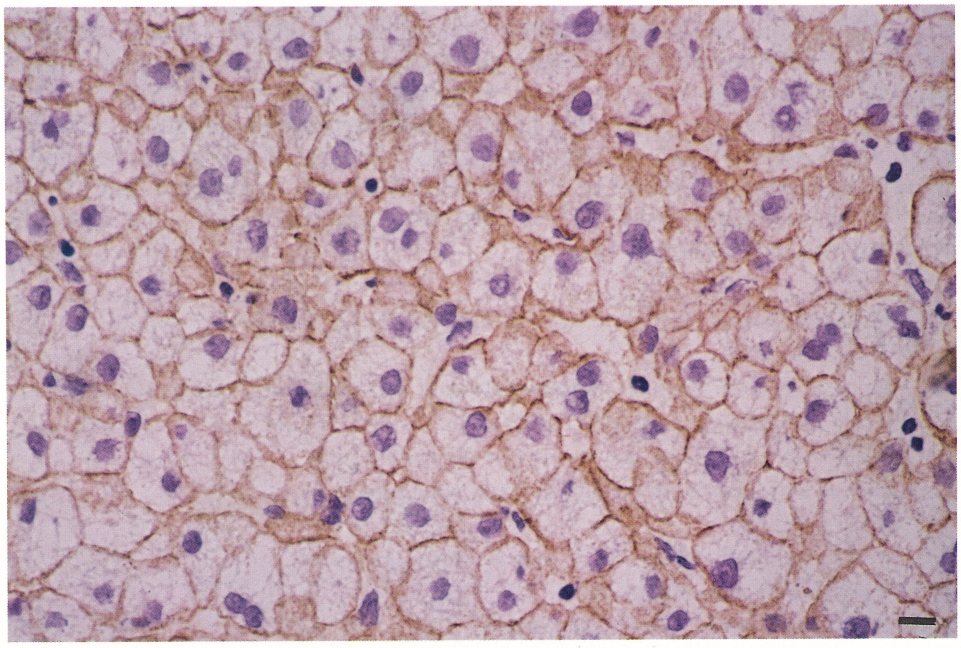

Fig 3. Frozen section of the liver stained for E-cadherin (ABC method).

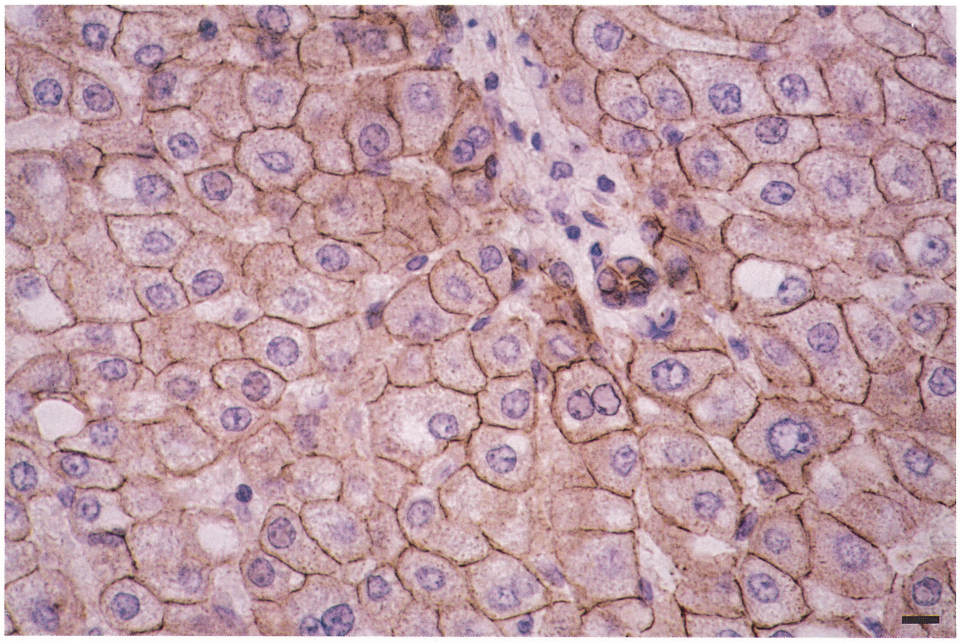

Fig 4. After microwave heating with 0.1% Tween 20 in a citrate buffer solution, formalin-fixed and paraffin-embedded liver tissue (24 hr).

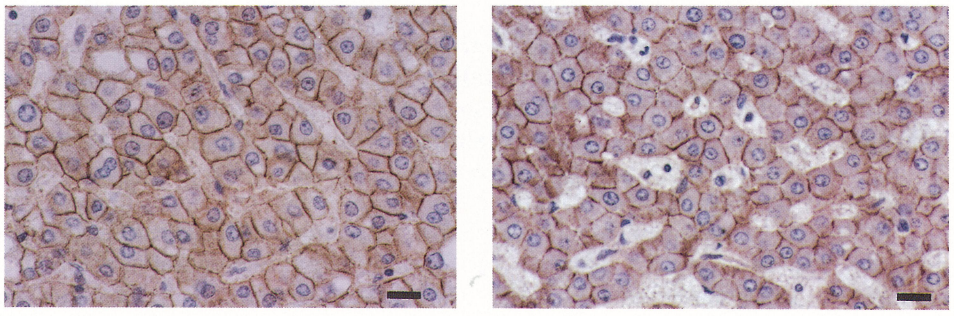

Fig 5. Formalin-fixed, paraffin-embedded sections of the liver immunohistochemically stained for E-cadherin after antigen retrieval procedures identical to that for Fig 4. (A) One stored for one month and one (B) for 10 years.

The length of time the tissues had been stored did not change the results and all the materials showed a similar immunoreactivity for E-cadherin. Note strong positive staining as strong as that with fresh materials is retained at the cell-to-cell juncsion between hepatocytes (Fig 5A, B).

DISCUSSION

Immunohistochemistry has, in recent years, established a strong foothold as one of the most useful ancillary procedures not only for its diagnostic and prognostic implications, but also as a technique for determining the biological significance of certain antigenic substances.

The cadherins are a multigene family of transmembrane glycoproteins, located on the plasma membrane and responsible for calcium-dependent intercellular adhesion.[11] E-cadherin is the major type expressed by almost all epithelial cells. Monoclonal antibodies capable of disrupting cell-cell adhesion were first raised and used to identify E-cadherin in human epithelial tissue in 1989.[10,12,13] Since then, many studies have been done regarding the relationship between E-cadherin expression and the metastatic potentials in human tumors,[3,6,14] and immunohistochemical techniques have been used for this purpose.

In immunohistochemistry using fixed materials, pretreatment with various proteases may be effective in retrieving the antigen. Success in such antigen retrieval depends upon the type of proteases, the duration of treatment, the type of dissolving solution, PH, and the duration of fixation. To date, E-cadherin has been constantly detected only with frozen fresh materials. Recently, using formalin-fixed materials, some authors have reported a successful retrieval of E-cadherin.[15–17] In our attempts, however, antigen retrievals of E-cadherin following exactly their procedures and those we tried without microwave oven heating or a special formula of submerging solution were neither complete or consistent. For instance, Sina *et al*[3] reported successful immunohistochemical reactivity of DECMA-1, a rat monoclonal antibody raised against murine E-cadherin, in formalin-fixed and paraffin-embedded colorectal cancer tissue using chymotrypsin. However, when we used DECMA-1, the background staining was strong and the cytoplasm was diffusely stained. Neither was their procedure effective with the antibody we used; *i.e.*, HECD-1. The diffuse cytoplasmic immunoreactivity may be due to passive diffusion or non-specific uptake of the antigen.

Recently, non-enzymatic antigen retrieval methods such as microwave oven heating in appropriate buffer,[18–20] alkaline hydrolysis in solutions containing NaOH,[21,22] formic acid etching of the section,[23] 4% aluminium chloride,[24] and refixation with precipitating zinc-containing solutions[25] have been shown to enhance immunostaining. However, we found some of these procedures to be unsuccessful for E-cadherin.

With our procedures reported in this communication, E-cadherin was perfectly and consistently detected in formalin-fixed and paraffin-embedded specimens. Even materials which had been stored for over 10 years could be utilized and linear immunopositivity was clearly revealed along the cell-to-cell junction between hepatocytes and between cholangiolar epithelia. The most important point for antigen retrieval with our procedure is microwave treatment with 0.1 mol/l Tween 20 in a citrate buffer solution. Neither citrate buffer alone nor Tween 20 in PBS retrieved antigenicity. Only with this combination could the E-cadherin antigen be retrieved. To the best of our knowledge, no one has used Tween 20 for the purpose of retrieving E-cadherin antigenicity.

The mechanism of the action of microwave oven heating for the retrieval of antigens is poorly understood. Shi *et al*[18] reported that tissues fixed in formalin for as long as two years could be stained for pancytokeratin and vimentin with microwave treatment in the presence of metal (zinc and lead) solutions. They hypothesized that the cross-linking effect of formaldehyde is altered by microwave heating. However, their theory does not explain our results in which immunoreactivity varied with the submerging solutions used

but not with the heating. Therefore, the immunoreactivity for E-cadherin antigen retrieved by microwave heating must depend upon some other factors. The submerging solutions, which contained metal, might have played an important role in the retrieval. Further studies are awaited in regard to this point. It should be also noted that with our procedure the duration of microwave heating may be constant and short even for materials fixed in formalin for longer periods.

In conclusion, we have reported a novel method for retrieving E-cadherin antigen by using 0.1 mol/l Tween 20 in a citrate buffer solution during microwave heating. We hope that the detection of E-cadherin by this method facilitates studies of cadherin biology and its diagnostic and prognostic implications.

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