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ORIGINAL ARTICLE

การย้อมอิมมูโนฟลูออเรสเซนต์ชนิดหลายสีในเนื้อเยื่อบางสไลด์เดียวกันของมะเร็งเต้านมชนิดลุกลาม: การศึกษาเบื้องต้น

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บทคัดย่อ

การแสดงออกของจีนต่างๆ ในตัวอย่างชิ้นเนื้อสามารถตรวจวัดได้โดยการย้อมชิ้นเนื้อที่แช่ในฟอร์มาลีนและฝังในพาราฟินด้วยเทคนิคอิมมูโนฮิสโตเคมีหรือย้อมชิ้นเนื้อสดด้วยเทคนิคอิมมูโนฟลูออเรสเซนต์ อย่างไรก็ตามการศึกษาหาตำแหน่งของแอนติเจนหลายชนิดในชิ้นเนื้อที่ตัดเป็นเนื้อเยื่อบางในสไลด์เดียวกันด้วยเทคนิคอิมมูโนฮิสโตเคมีนั้นเป็นไปได้ยากและผลการย้อมไม่ดีทำให้แปลผลได้ยาก อีกทั้งต้องอาศัยขั้นตอนการกู้คืนแอนติเจนที่เหมาะสมซึ่งแอนติเจนเหล่านั้นถูกทำลายหรือถูกบดบังไปจากการแช่ในฟอร์มาลีน ในขณะที่การย้อมด้วยเทคนิคอิมมูโนฟลูออเรสเซนต์สามารถทำได้และให้ผลการย้อมดี แต่อย่างไรก็ตามการเก็บชิ้นเนื้อสดสำหรับย้อมอิมมูโนฟลูออเรสเซนต์นั้นค่อนข้างลำบากและยุ่งยากจากข้อจำกัดของทั้งสองเทคนิค นี้จึงได้มีการศึกษาพัฒนาเทคนิคต่างๆเพื่อลดข้อจำกัดดังกล่าว ในการศึกษาครั้งนี้ทางกลุ่มผู้ศึกษาได้ทำการพัฒนาเทคนิคการกู้คืนแอนติเจนสำหรับการย้อมอิมมูโนฟลูออเรสเซนต์ในการตรวจหาแอนติเจนสามชนิดที่เป็น biomarker (Ki-67, Her-2 protein, E-cadherin) ในชิ้นเนื้อมะเร็งเต้านมชนิดลุกลามที่แช่ในฟอร์มาลีน ฝังในพาราฟินและทำเป็นเนื้อเยื่อบางในสไลด์เดียวกัน จากการศึกษาพบว่า การกู้คืนแอนติเจนโดยใช้สารละลาย 0.05 % citraconic anhydride, pH 7.4 ในไมโครเวฟให้ผลการย้อมที่ดีทั้งสามชนิดที่ย้อมในสไลด์เดียวกันแสดงให้เห็นว่าเทคนิคนี้เหมาะในการศึกษาตำแหน่งของแอนติเจนหลายชนิดในสไลด์เดียวกัน นอกจากนี้ยังเหมาะกับตัวอย่างชิ้นเนื้อที่เจาะด้วยเข็มมาตรวจหาแอนติเจนหลายชนิดแต่ชิ้นเนื้อไม่พอสำหรับการตัดหลายๆ สไลด์

คำสำคัญ: การกู้คืนแอนติเจน, Citraconic anhydride, อิมมูโนฟลูออเรสเซนต์, อิมมูโนฮิสโตเคมี

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Multiple immunofluorescence labeling of the same section of invasive ductal breast cancer: A preliminary study

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Abstract

The expression of candidate genes in tissue samples were investigated by using immunohistochemical study of formalin-fixed paraffin-embedded (FFPE) tissue or immunofluorescence labeling of cryosections. However, the study of co-localization of multiple antigens in the same section by immunohistochemical labeling is difficult to evaluate and involves antigen retrieval step to unmask antigen whereas immunofluorescence labeling has the capability for multiple labeling with higher resolution. Nevertheless, the handling of fresh tissue as immunofluorescence labeling is difficult. Thus, both methods have limitations as research tools. In this study, we optimized an antigen retrieval method for high-resolution immunofluorescence labeling of FFPE invasive breast cancers using three different biomarkers (Ki-67, Her-2 protein and E-cadherin). Citraconic anhydride solution at 0.05 % pH 7.4 in a microwave oven seemed to work well for multiple antibodies for co-localization of multiple antigens in the same section. In addition, this method is suitable for core needle biopsy which obtained small amount of tissue. Therefore, it is not enough for preparation of multiple sections.

Key words: Antigen retrieval, Citraconic anhydride, Immunofluorescence; Immunohistochemistry

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Introduction

Immunohistochemistry (IHC) is an important tool for diagnosis and research in the field of pathology. However, limitations of this technique are divided into three major categories. First, it is primarily used to demonstrate one protein at a time. The examination of co-localization for two antigens in the same section is not satisfied when using combined IHC. Second, the resolution of antigen localization is limited due to the chromogenic substrate precipitation and the thickness (3 - 4 μm) of the sections imaged in the light microscope. Third, chromogenic systems saturate easily which restricts semi-quantitative analysis. On the other hand, immunofluorescence (IF) labeling has the capability in multiple labeling with higher resolution due to the direct conjugation of fluorophores to antibody[1, 2]. However, immunofluorescence labeling is unsuitable for formaldehyde-fixed and paraffin-embedded (FFPE) specimens because of the masking of tissue antigens by protein cross-linking through formaldehyde reaction with the protein amino groups. The loss of antigenicity is often caused by sequential processes of fixation, dehydration and embedding in paraffin-embedded tissue leading to the preclusion of effective immunological analysis[3]. For this reason, various unmasking antigens referred to as antigen retrieval (AR) methods were developed. There are widely used methods for retrieving antigens for FFPE tissues, such as proteolytic digestion [4-6], heat-induced treatment[7-12], and a mixed method of both treatments[13]. Variation in the solution media, buffer equilibrium, temperature, and heat source have been used to adjust for successful AR-IHC by different laboratories. Recently, Namimatsu reported a novel retrieval protocol; citraconic anhydride (a reversible protein cross-linking agent) solution with heating under optimal condition was able to satisfactorily retrieve a wide variety of antigens for IHC[14]. In our study, we used this new AR solution to unmask antigen compared with conventional AR solution (10 mM citrate buffer, pH 6) before detection by multiple immunofluorescence staining and described a procedure for simultaneous immunofluorescence localization of three different biomarkers for breast cancer including Ki-67, Her-2 protein, and E-cadherin within the same tissue section. Using this method, we demonstrated that multicolour immunofluorescence imaging of FFPE material is readily achievable and that this method provides excellent images.

Materials and Methods

Eleven cases of human breast tissue diagnosed with invasive ductal breast cancer and graded by immunoperoxidase (IP) staining for Ki-67, Her-2 protein, and E-cadherin (**Table 1**) were obtained from the Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand. The specimens were fixed in 10% buffer formalin and embedded in paraffin for routine diagnosis. The sections were cut into a 3 ?m thickness, attached to silan-coated slides, then were deparaffinized, rehydrated in a graded series of ethanol and soaked in 3% hydrogen-peroxide in methanol for 5 min to block endogenous enzyme activity, and finally washed for 5 min with phosphate buffer saline (PBS).

Table 1 List of specimens and immunoperoxidase (IP) staining score.

Cases	IP staining score		
	Her-2	Ki-67	E-cadherin
1	2+	1+	1+
2	2+	1+	3+
3	3+	4+	2+
4	2+	3+	1+
5	3+	4+	3+
6	2+	4+	2+
7	2+	4+	3+
8	1+	4+	3+
9	3+	3+	2+
10	2+	1+	2+
11	1+	4+	1+
Average	2.10 ± 0.70	3.0 ± 1.34	2.09 ± 0.83

New antigen retrieval (AR) method

After washing in PBS, the sections were subjected to the antigen retrieval solution, 0.05% citraconic anhydride, pH 7.4. The soaked slides were then heated by microwave (Electrolux model EME2820) treatment at 100oC for 10 min. Subsequently, sections were allowed to cool for 20 min at room temperature before rinsed in running tap water, followed by distilled water and PBS.

Conventional antigen retrieval (AR) method

After washing in PBS, the sections were subjected for the antigen retrieval by immersing into 10 mM citrate buffer, pH 6 followed by microwave treatment at the heat level 10 for 3 min and level 3 for 10 min. After the completion of cycle, sections were allowed to cool for 20 min at room temperature and then rinsed in running tap water, distilled water, and PBS.

Immunofluorescence method

After pretreatment, the sections were treated with PBS containing 3% normal horse serum at room temperature for 20 min, and then incubated with combination of primary antibodies: mouse anti human Ki-67, rabbit anti- human Her-2 protein, and mouse anti-human E-cadherin (DAKO, Glostrup, Denmark), for 1 h at room temperature. After washing with PBS, the sections were incubated with combined solution of swine anti rabbit immunoglobulin antibody conjugated with FITC (DAKO, Glostrup, Denmark) and goat anti mouse immunoglobulin antibody conjugated with Alexa (Invitrogen, Eugene, Oregon, USA) for 1 hour. After washing with PBS, the sections were mounted with mounting media.

Interpretation

Interpretation of immunofluorescence stained sections were performed blindly without the knowledge of AR methods used. We defined the immunostaining of Ki-67 by the mean percentage of nuclear staining of tumor cells and classified as 0 %, -; <5 %, +/-; 5-25 %, 1+; 26-50 %, 2+; 50-75 %, 3+; and >75 %,4+. For Her-2, membrane staining intensity and number of invasive tumor cells staining were evaluated scoring 0-3+ as illustrated by the HercepTest kit scoring guidelines[15]. No staining observed classified as 0; faint membrane staining and stained in only part of the membrane, 1+; non-uniform or week staining circumferential distribution is > 10 % of invasive tumor cells, 2+; and uniform intense membrane staining of 30 % of invasive tumor cells, 3+[16]. Scores of 0 or 1+ were considered negative for Her-2 over expression; scores of 2+ were considered weakly positive; and scores of 3+ were considered strongly positive[15]. For E-cadherin, immunostaining was also divided into 4 categories: strong membranous staining in more than 80 % of cells (3+); homogenous positive tumor cells (more than 50 %-80 % positive tumor cells; 2+); heterogeneous staining (20 %-50 % positive tumor cells; 1+); negative (less than 20 % positive tumor cells or no evidence of membrane staining;-)[17]. The average score of each antibody were expressed in mean ± S.D.

Statistical analysis

Statistical analysis was performed using statistical test by Sigma stat version 2.0. Normality test was checked by Kolmogorov test. Independent-sample t-test (if the distribution was normal) or a Wilcoxon signed rank test (if the distribution was not normal) was used to observe statistical differences between AR methods in each antibody. The level of statistical significance was set at 0.05.

Table 2 Comparison of immunofluorescence staining by different antigen retrieval methods.

	IF (0.05 % citraconic anhydride)			IF (conventional method)		
Cases	Her-2	Ki-67	E-cedherin	Her-2	Ki-67	E-cedherin
1	2+	2+	2+	2+	1+	1+
2	2+	1+	3+	2+	1+	3+
3	3+	3+	2+	2+	1+	1+
4	3+	2+	1+	2+	1+	1+
5	2+	3+	3+	2+	2+	2+
6	3+	3+	1+	2+	2+	1+
7	2+	3+	2+	2+	1+	2+
8	-	2+	3+	-	-	3+
9	3+	3+	2+	3+	3+	2+
10	1+	-	2+	1+	-	1+
11	1+	4+,	1+	-	3+	-
Average	2.00 ± 1.0	2.36 ± 1.23	2.00 ± 0.77	1.64 ± 0.92	1.36 ± 1.03	1.55 ± 0.93

Results

Table 2 summarizes the results of immunofluorescence staining with three antibodies by different AR methods in the same section. Immunoperoxidase staining with three antibodies in each section showed in **Figure 1.** The localization of Her-2 protein showed plasma

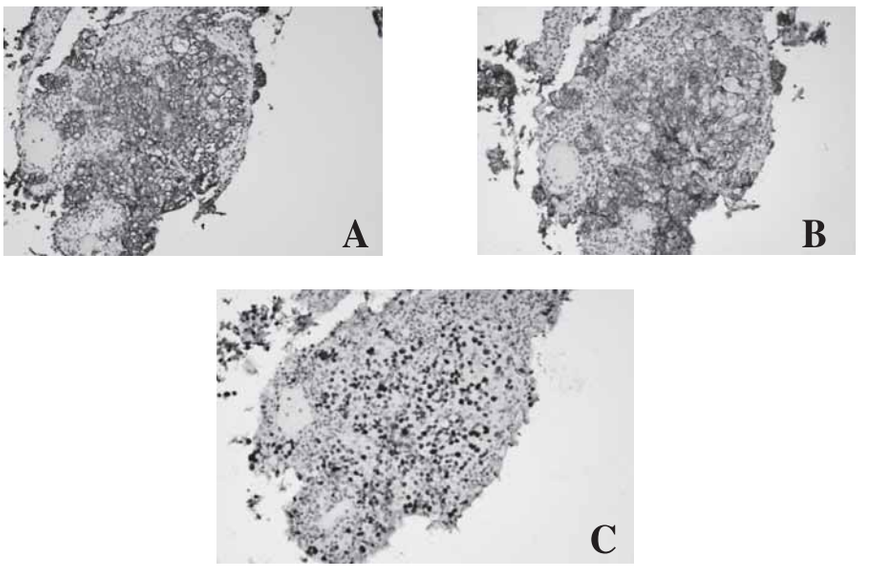

Figure 1 Representative micrographs showing IHC staining of formalin-fixed, paraffin-embedded breast tissue. Her-2 protein showed plasma membrane staining (A), E-cadherin showed deposition on plasma membrane (B) and Ki-67 showed nuclear staining (C). 20X

membrane staining **(Figure 1A)**, E-cadherin showed deposition on plasma membrane **(Figure 1B)** and Ki-67 showed nuclear staining **(Figure 1C).** Immunofluorescence staining using Ki-67 staining showed nuclear positive of red color while E-cadherin was stained red color at plasma membrane **(Figure 2A)**. Her-2 antibody showed green color staining of the plasma membrane of tumor cell, consistent with the expected location of the Her-2 protein **(Figure 2B)**. When combined staining, the result showed co-localization of Her-2 protein and E-cadherin

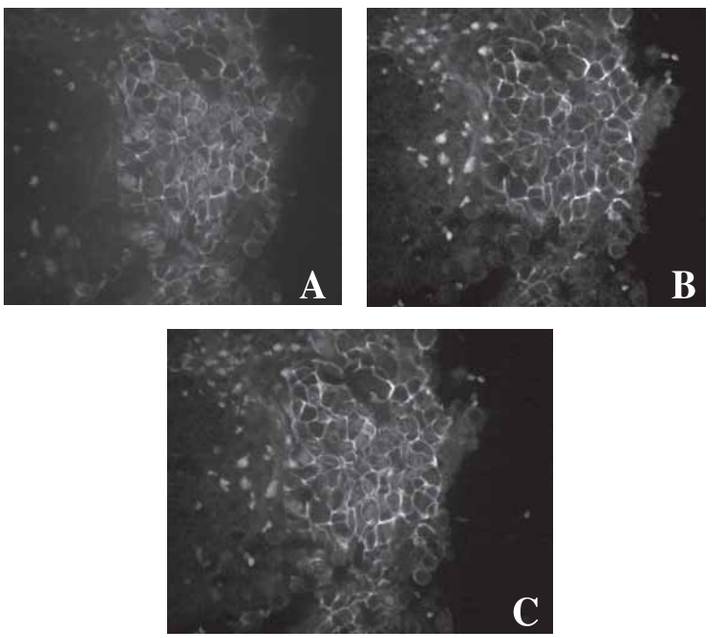

Figure 2 Representative micrographs showing IF staining of formalin-fixed, paraffin-embedded breast tissue. Ki-67 and E-cadherin detected by anti mouse IgG-conjugated Alexa (A). Her-2 protein detected by anti rabbit IgG-conjugated FITC (B). Combined detection of Ki-67, E-cadherin and Her-2 protein in the same section (C). 20X

in plasma membrane of the same tumor cell in which orange color was detected (red plus green color). In different AR methods, we found that average scores of immunostaining of three antigens obtained by AR with 0.05 % citraconic anhydride solution were similar to those of IP method, which is a routine method for diagnostic tool **(Table 3)**. On the other hand, conventional AR method revealed the lower average scores of

Table 3 Comparison of the average scores of three antibodies staining by IF method with different antigen retrieval conditions to IP method.

Primary antibodies	Average score		
	IP	New IF	Conventional IF
Anti-Her-2	2.10 ± 0.70	2.00 ± 1.00	1.64 ± 0.92
Anti-Ki-67	3.00 ± 1.34 *	2.36 ± 1.12	1.36 ± 1.03 *
Anti-E-cadherin	2.09 ± 0.83	2.00 ± 0.77	1.55 ± 0.93

* $P < 0.05$

immunostaining than those of IP method, especially Ki-67 antibody was statistically significant ($P < 0.05$). These antigens can be divided into two groups according to localization of antigens expressed including nuclear antigens (Ki-67) and membrane antigens (E-cadherin, Her-2). We found the membrane antigens were effectively unmasked by using both solutions. Ki-67 is a difficult-to-detect antigen in which the antigenicity can be restored by 0.05 % citraconic anhydride solution.

Discussion and Conclusion

We reported a new antigen retrieval technique that allows multiple immunofluorescence labeling of formalin-fixed paraffin-embedded tissue samples. Antigen retrieval is a very important step for immunostaining because it can break the protein cross-links formed by formalin fixation and thereby uncover hidden antigenic sites. However, different antigens are suitable for different antigen retrieval methods. Previously reported, high temperature (120°C) or strong alkaline hydrolysis can reverse the cross-linking of protein produced by formaldehyde fixation [18]. In the present study, we used citraconic anhydride solution in microwave compared with the conventional methods (10 mM citrate buffer, pH 6 in microwave treatment) to detect three biomarkers for diagnosis of an invasive breast cancer namely Ki-67, E-cadherin, and Her-2 protein in the same section. The results showed that 0.05 % citraconic anhydride solution unmasked the three antigens in the same section of paraffin-embedded breast tissue whereas conventional method was less efficient to unmask these antigens, especially Ki-67 (nuclear antigen). This result agreed with the report by Namimatsu et al., who used 0.05 % citraconic anhydride solution and heat under an optimal condition which was able to satisfactorily unmask a wide variety of antigens for IHC such as CD4, cyclin D1, granzyme β, and PAR4 antigenic determinants[14]. Shi and coworkers employed 0.05 % citraconic anhydride

as the AR solution and heat treatment in comparison with different methods for immunostaining of retinoblastoma protein (pRB) in FFPE tissue sections. They found that this protocol has several advantages including superior morphological preservation, greater reproducibility, and more intense staining after retrieval[19]. The mechanism underlying this new AR method is believed to be the reaction of formaldehyde mainly with the the lysyl residues and the formation of intra-molecular cross-links[20]. The free amino groups of proteins form aminomethylol groups and then combine with other functional groups, such as phenolic, imidazole, and indole groups to form methylene bridges. Citraconic anhydride reacts with the free amino groups of proteins and replaces the positively charged NH3+ groups of lysyl residues with negatively charged carboxyl groups. At neutral pH 7.4 in hot water, adduct of citraconic anhydride and amines will undergo slow hydrolysis, which will liberate the original amines[21].

In conclusion, 0.05 % citraconic anhydride solution is the best retrieval solution for unmasking several antigens such as membrane antigen (E-cadherin, Her-2) and nuclear antigen (Ki-67) and useful for studying the multiple co-localizations of biomarker in cancer tissue using immunofluorescence method. In addition, background staining in IF method was clear.

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