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Immunocytochemical detection of prognostic markers in breast cancer; technical considerations

V. KUENEN-BOUMEESTER, A. M. TIMMERMANS,
E. M. C. A. DE BRUIJN AND S. C. HENZEN-LOGMANS
Department of Pathology, University Hospital Rotterdam/Daniel den Hoed Cancer Centre, Rotterdam, The Netherlands

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The purpose of this study was to establish a good technical procedure for immunocytochemical (IC) staining of prognostic markers in breast cancer specimens. The influence of various preparation, fixation and storage methods on ER, P53 and Ki-67 IC staining was assessed, using cells of two breast cancer cell lines T47D (ER/P53$^+$) and ZR-75-ER (ER$^+$, P53$^-$). In addition we searched for a suitable transport medium. Depending on the technical procedure, great variations in expression of the tested antigens were found. Cytospins fixed and stored according to the Abbott method gave the best results. Histocon appeared to be the medium of choice. A good concordance of IC and immunohistochemical (IH) results was found when the adopted method was tested on material of 10 breast cancers. This study underlines the importance of quality controlled standardization of cell processing, fixation and storage of fine needle aspiration (FNA) aspirates in order to obtain reproducible and consistent IC results.

Keywords: immunocytochemistry, fine needle aspirates, breast cancer, prognostic markers, cell preparation

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INTRODUCTION

The use of immunocytochemistry in the examination of fine needle aspirates (FNAs) of breast tumours has enhanced the diagnostic possibilities, especially with regard to the assessment of prognostic markers. Until now most prognostic markers, like oestrogen

Correspondence: V. Kuenen-Boumeester MD, Department of Pathology, Dr Daniel den Hoed Cancer Centre, PO Box 5201, 3800 AE Rotterdam, The Netherlands.
E-mail: Kuenen-Boumeester@padh.azr nl

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receptor (ER), Ki-67 and P53, have been biochemically and/or immunohistochemically assessed on tissue specimens obtained at surgery. However, early detection of breast cancer through breast cancer screening programmes has led to reduction of the size of breast carcinomas[1] and therefore to a reduction in the amount of tissue available for diagnostic and prognostic information. In addition, new treatment modalities, such as neo-adjuvant chemotherapy for T4 tumours, necessitate the maximum yield of information from cytological specimens. Good correlations between biochemical, histological and cytological assays have been reported[2–4]. Nevertheless discrepancies, due to heterogeneity of the tumour and to methodological differences, may exist. Here we describe a study to establish a suitable technical procedure for immunocytochemistry (IC) of FNA material, especially with regard to nuclear antigens. We compared the influence of various preparation, fixation and storage methods (study A). We made an assessment of the influence of transport media on quality of immunostaining (study B). Finally, we compared IC staining of cell material and frozen tissue samples of corresponding breast cancers, using the cell material processing technique and transport medium of choice (study C).

MATERIALS AND METHODS

Study A: the influence of various preparation, fixation and storage methods on IC staining results

Cell lines T47D (ER^+, $P53^+$) and ZR-75-ER (ER^+, PR^-) were used for this study. Cells were harvested, pelleted by centrifugation and resuspended in RPMI 1640 medium.

I. Cell preparation. Two methods of cell preparation were compared.

1. A standardized method used to prepare serous effusions as described before[5]. In short, cell suspensions were diluted with 1% bovine serum albumin (BSA)/PBS, pH 7.4. After centrifugation (5 min at 738 ***g***) and decanting, the pellet was lysed and fixed at 4°C for 10 min by adding 2.5 ml lysis buffer containing isotonic ammonium chloride (4.5 g NH_4Cl (Merck, Darmstadt, Germany) and 0.5 g $KHCO_3$ (Merck)) and 0.0186 g EDTA (Merck) dissolved in 500 ml aqua bidest pH 7.4; and 2.5 ml 4% paraformaldehyde (PFA; Merck). The cells were rinsed in PBS, followed by centrifugation and decanting. Then the pellet was resuspended and diluted in BSA 1% in PBS and adjusted to a cell concentration of 1×10^6 cells/ml using a Bürker cell counting chamber (Optik Labour, Bad Homburg, Germany) to obtain reproducible specimens with optimal cell density. Finally, cytocentrifuge samples were made by centrifugation at 55 ***g*** for 5 min using a Shandon cytospin 2 (Astmoor, Runcorn, UK). The processed slides were air-dried for a maximum period of 5 min.

2. The same as protocol 1, but without lysing and fixation of the pellet.

II. Fixation and storage. Different procedures were compared: (i) no fixation of processed slides. The slides were kept at room temperature overnight; (ii) fixation of cytospins with 4% paraformaldehyde followed by methanol/acetone at −20°C (Abbott method); (iii) fixation by methanol/acetone only; (iv) cytospins directly stored at −80°C and fixed afterwards according to either method ii or iii (see Table 1 for exact procedures). The different protocols of studies A and B were carried out at the same time, under the same conditions. The experiments were repeated at least three times, sometimes more, again under the same conditions, but on different days, using the same cell lines. For reasons of clarity we have taken the average outcome of the different experiments for Table 2.

III. Immunocytochemistry. The non-fixed slides were fixed in acetone for 10 min prior to

Table 1. Fixation and storage protocols (study A II)

Method	Fixation/storage	Temperature	Time
i	Air-dried	RT	Overnight
ii	Paraformaldehyde 4%	RT	10 min
	PBS 3×		10 min
	Methanol	−20°C	3 min
	Acetone	−20°C	1 min
	Storage medium	−20°C	
iii	Methanol	−20°C	3 min
	Acetone	−20°C	1 min
	Storage medium	−20°C	
iv	Directly stored at	−80°C	
	Direct fixation upon thawing followed by method ii or iii		

RT, Room temperature.

incubation and together with the prefixed cytospins rinsed in PBS pH 7.4, followed by preincubation using 1% BSA diluted in PBS for 10 min. The antibodies raised against ER (1D5; Dako, Glostrup, Denmark), Ki-67 (MIB-1; Immunotech/Coulter, Westbrook, ME) and P53 (1801; Oncoscience, Cambridge, MA) were used for this study and diluted in 1% BSA in PBS. Incubation was carried out for 60 min at room temperature. The APAAP technique (Dako; working dilution 1:20) was applied after incubation with the linking antibody (working dilution 1:20 in 5% normal human serum (NHS) in PBS) for visualizing the primary antibody. All immunostained slides were counterstained with Mayer's haematoxylin (Klinipath, Duiven, Holland) for 1 min. Slides of cell lines were included as positive controls; as negative controls the primary antibody was replaced with 1% BSA diluted in PBS in each run.

Study B: the influence of transport media on IC results

The following media were compared.

1. RPMI 1640 containing phenol red, 21 mM HEPES, 10 mM $NaHCO_3$, 4 mM glutamine, 100 U/ml streptomycin, 100 mU/ml penicillin, 45 μg/ml gentamycin, 10 μg/ml porcine insulin, 5 μg/ml insulin, 2.5 μg/ml oestradiol and 10% bovine calf serum (heat-inactivated).
2. RPMI 1640 supplemented with 10% fetal calf serum (FCS) heat-inactivated, 20 000 IE/*l* penicillin/streptomycin, 20 000 IU/*l* heparin sulphate, 300 mg/*l* L-glutamine (GIBCO BRL, Breda, The Netherlands).
3. PBS–BSA 1% pH 7.4.
4. Histocon (Polysciences, Warrington, PA).

Cell material of histologically proven breast carcinomas was obtained by scraping a scalpel blade firmly against the carcinoma and by directly depositing the material in the media. Cell specimens were prepared according to the best method of Study A (see Results). The influence of the various media on IC staining was determined.

IV. Evaluation of immunostaining. The assessment of staining was done by V.K.-B., without prior knowledge of the protocols used.

Study C: Comparison of immunostaining of cytological and corresponding histological specimens

Frozen tissue samples and cell scrapings of 10 histologically proven breast carcinomas were obtained. Cryostat sections (4 μm thick) were fixed in formalin 4% for 10 min. Cell material was suspended in the chosen transport medium of Study B and cytospins were prepared according to the adopted method of Study A (see Results). Immunostaining of cytospins and cryostat sections was performed using the above mentioned technique, using the antibodies anti-ER, anti-P53 and Ki-67.

Quantification

Frozen material. The percentage of IH-assessed ER and P53-stained tumour cells was calculated by scoring the number of positive cells semiquantitatively in a total of 300 cells in three different areas of the tumour section[6]. The Ki-67 score was assessed by counting 300 cells in the areas with the highest activity as described previously[6,7].
Cell material. The ER, Ki-67 and p53 score was assessed by counting at random 500 cells as described before[7]. The IC and IH scores were compared.

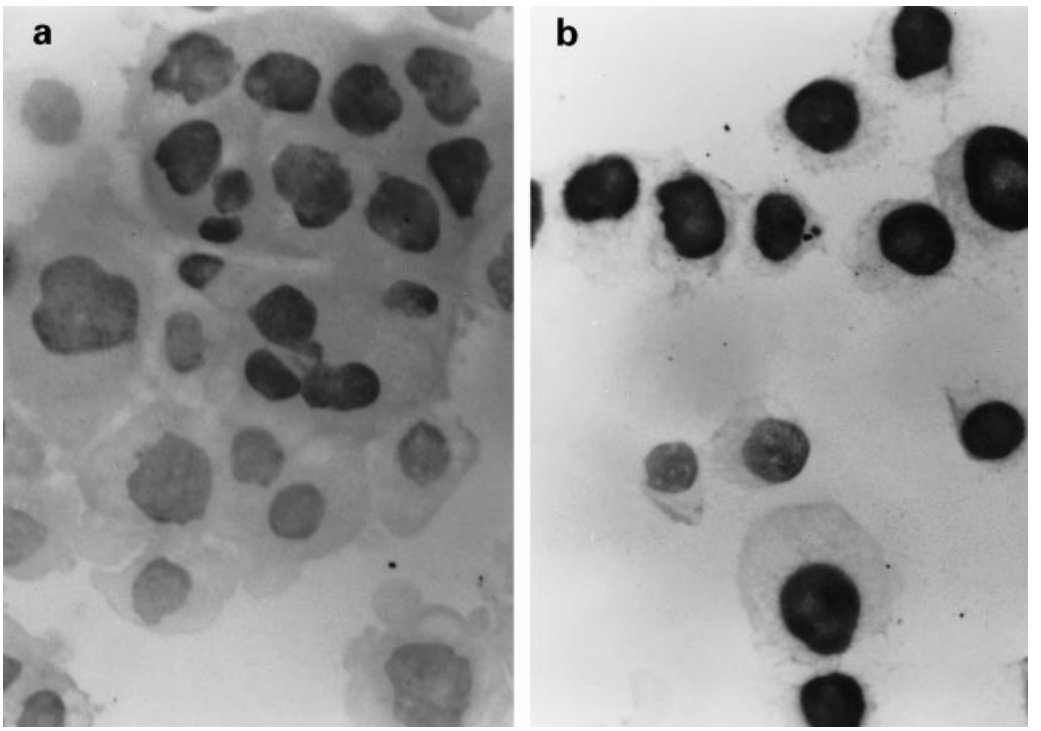

Figure 1. Influence of cell processing on P53 immunostaining. (a) Prefixation of suspension with 4% paraformaldehyde followed by fixation and storage of the air-dried cytospin according to the Abbott method. (b) Fixation and storage of air-dried cytospin according to the Abbott method.

RESULTS

Study A

Material processing and fixation

Variations in staining intensity between the two preparation methods were seen. In general, cell preparation method A.I.1 (prefixation of the cell pellet) showed little or no nuclear staining, in contrast to the strong staining of method A.I.2 (fixation of cytospins) (Figure 1a,b). A rapid loss of antigenicity was seen when the cytospins were kept at room temperature for longer than 5 min. Direct freezing of the cytospins before fixation followed by direct fixation upon thawing according to the various tested methods gave inconsistent results. Cell processing method A.I.2, followed by fixation and storage of the cytospins according to the Abbott protocol, gave the best results (demonstrated in Figure 2a,b and Table 2).

Study B

Morphology and immunoreactivity of cells was best preserved when transported in either Histocon or 1% BSA (Table 3).

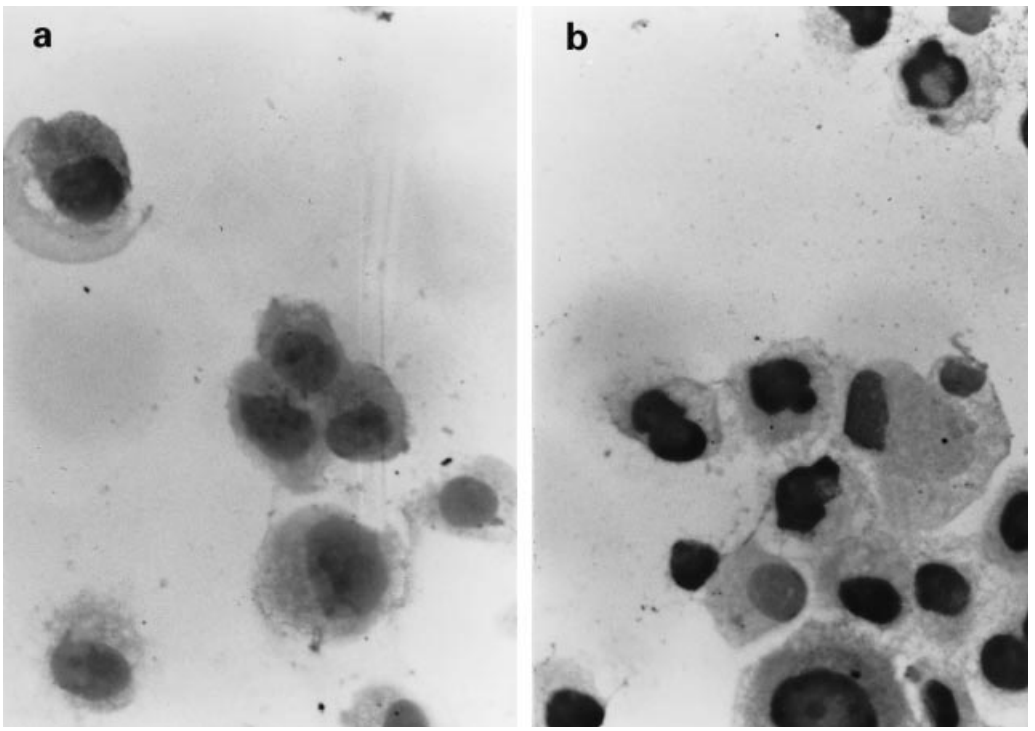

Figure 2. Influence of different storage and fixation methods on P53 immunostaining
(a) Storage at –80°C, followed by fixation with paraformaldehyde 4%. (b) Fixation and storage of air-dried cytospins according to the Abbott method.

Table 2. Immunostaining of cell specimens of two cell lines using various fixatives, prepared according to preparation method A.I.2

	ER		Ki-67		P53	
	A	B	A	B	A	B
Fixation/storage						
i	−	+/−	−	+/−	−	+/−
ii	++	++	++	++	−	+++
iii	+/−	+	+/	+	−	+
iv + ii*		+/−		+/−		+/−
iv + iii*		−		−		−

A, Cell line ZR-75-ER (ER^+, $P53^-$).
B, Cell line T47D (ER^+, $P53^+$).
Method i, air-dried/room temperature overnight; method ii, 4% paraformaldehyde + methanol/acetone (Abbott method); method iii, methanol/acetone; method iv, −80°C.
*Only tested with cell-line T47D.

Study C

The 10 carcinomas demonstrated a good correlation between the IH staining of frozen material and IC staining of corresponding cell material, suspended in Histocon and processed according to method A.I.2 and fixed following the Abbott method (Table 4).

DISCUSSION

In the future IH examination of material obtained at surgery may partly be replaced by IC examination of needle aspirates, and therefore methods of investigating prognostic indicators on cytological specimens will expand. In the literature both smears[8,9] and cell blocks[8,10] are used for IC, but we prefer to use cytospins, even though the preparing of cytospins is more time consuming in comparison with smears. The fact that more preparations can be made under standardized conditions, that less reagent is needed for the

Table 3. A comparison of immunocytochemistry results of cell material of a histologically proven ER/P53 † breast cancer, suspended in different transport media and prepared according to the adopted method

	Medium 1	Medium 2	1% BSA	Histocon
ER	+/−	+/−	++	++
P53	−	−	+	+
Ki-67	+/−	−	+	+

Table 4. Percentages of positive immunohistochemically (IH)/immunocytochemically (IC)-stained cells in frozen tissue samples and corresponding cytospins of 10 breast cancers

	ER, %		P53, %		Ki-67, %	
Sample	IH	IC	IH	IC	IH	IC
1	40	50	< 10	< 10	50	50
2	100	100	100	100	50	40
3	0	0	0	0	40	50
4	60	60	< 10	< 10	40	40
5	0	0	90	90	80	70
6	80	70	< 10	0	30	20
7	30	40	0	< 10	0	< 10
8	50	30	0	0	40	20
9	80	80	30	30	10	< 10
10	< 10	< 10	0	0	10	< 10

staining, and that the reading of the cytospin is easier due to concentration of cells, makes it altogether worthwhile. In spite of these considerations our cytospins processed for ER and PR analysis have shown inconsistent results. This study was undertaken to determine the cause of these inconsistencies and to determine what preparation and fixation method was the best for general use.

In an earlier study[11] we demonstrated that with regard to serous effusions, the most consistent immunostaining was found when lysis and prefixation of the material was applied (see method A.I.1). In this study, however, cell processing method A.I.1 gave inferior IC results when compared with method A.I.2 (see Figure 1a,b). This may well be explained by the fact that nuclear antigens, such as we studied, require different methods of preparation in comparison with cytoplasmic and membrane-bound antigens, in general identified in serous effusions. Therefore, prefixation of the pellet in method A.I.1, excellent for IC staining of effusions, gave poor results when used for staining of nuclear antigens, possibly due to inactivation of the epitopes. Since we found antigen retrieval methods such as pretreatment with microwaves difficult to standardize, we prefer to use method A.I.2. In this method the prefixation of the cytospin is kept short (10 min) and is followed by fixation in acetone (Abott method). This method is well known in general practice and easy to apply.

In keeping with other reports[12,13], we observed a rapid deterioration of the immunoreactivity of the nuclear antigens after storage of air-dried slides at room temperature for more than 5 min. In contrast to these findings, Suthipintawong *et al.*[9], who kept the air-dried smears at room temperature for up to 1 week, did not observe any deterioration of IC staining. Here again the localization of the studied antigens, nuclear antigens *vs* cytoplasmic/membrane-bound antigens, respectively, may well explain this discrepancy.

In the experience of Dowell *et al.*[12] and Burton *et al.*[13] staining results were not affected if the specimens were stored unfixed at −70°C and fixed immediately upon thawing. Applying this method, we observed negative/weak staining (Figure 2a). A possible

explanation for these negative results may be that due to our working situation (freezer located in other room) the air-dried cytospins were not directly frozen, nor fixed immediately upon thawing, resulting in loss of antigenicity. The fact that delay of freezing of the material has a negative effect on IC staining was confirmed by the findings of Coddington *et al.*[14].

Numerous suspensions are recommended for preservation of cells[15,16]. In our laboratory we employed RPMI culture media. We found that cell scrapings of breast cancers suspended in these media showed inferior IC results compared with IH of frozen tissue samples. For this reason we evaluated the influence of a number of media and found that preservation of cell morphology and immunoreactivity was best when suspended in either 1% BSA or Histocon. In agreement with Burton *et al.*[13], we recommend immediate preparation of the cells (within 1 h) to avoid a decrease in antigenicity. Since 1% BSA is easily contaminated by microorganisms and fungi, the use of this medium is not recommended.

By showing the influences of different preparatory, fixation and storage methods we have emphasized the need for quality controlled, standardized methods of material processing and fixation of FNA material for IC staining, especially with regard to nuclear antigens. Taking these technical problems into consideration, we find IC of FNA material a reliable technique for the assessment of prognostic markers in breast carcinomas.

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