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Involucrin expression in breast carcinomas: an immunohistochemical study

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Abstract. The expression of involucrin, a structural component of the envelope of mature squamous epithelium, was studied in 166 paraffin-embedded breast carcinomas. In 41 cases (24.7%) involucrin-positive, light microscopically non squamous tumour cells were detected. The number of involucrin-positive tumour cells varied considerably from case to case. For further characterization, involucrin-positive cases were studied using monoclonal antibodies to various cytokeratins (PKK1, EAB 903, EAB 904) and, in selected cases, double immunostaining with antibodies to cytokeratins and involucrin were performed. Coexpression of involucrin and cytokeratins demonstrated by PKK1 was seen in all tumour cells, whereas coexpression of involucrin and cytokeratins detected by EAB 904 was only seen in single and scattered cells in a few cases. Cytokeratins detected by EAB 903 were not coexpressed with involucrin in our cases. Our results indicate heterogeneity of cytokeratins in breast carcinomas and suggest a dissociation in the regulation of involucrin and cytokeratin expression.

Key words: Breast carcinoma – Cytokeratin – Involucrin – Immunohistochemistry

Introduction

Involucrin (INV) is a cytoplasmic 92-kDa protein isolated from cultured human epithelial cells (Rice and Green 1979). Acting as a substrate for transglutaminase, INV becomes cross-linked into a submembranous envelope to form insoluble polymers (Rice and Green 1979; Simon and Green 1984, 1985, 1989; Thacher and Rice 1985; Michel et al. 1987). INV is thus a marker for terminal differentiation of keratinocytes in stratified squamous epithelia. The protein is distributed in the granular layer of the epidermis or adjacent upper spinous layer (Warhol et al. 1982; Said et al. 1984; Walts and Said 1985; Itoiz et al. 1986; Sumitomo et al. 1986; Kamino et al. 1988) and can be visualized in paraffin-embedded tissue sections by immunohistochemical procedures (Warhol et al. 1985).

Many reports have described the immunohistochemical detection of INV in paraffin sections of normal and neoplastic tissues. It has been found in skin tumours of epithelial and adnexal differentiation and appears to serve as a valid marker of squamous differentiation (Kanitakis et al. 1986). INV has also been demonstrated in nasopharyngeal carcinomas (Kamino et al. 1988) and in various lung tomours with squamous differentiation (Said et al. 1983; Mayall et al. 1992). Specimens of normal thyroid, follicular adenomas, follicular and medullary carcinomas were found to be INV negative, but INV was observed in a few tumour cells in papillary carcinoma of the thyroid (Rice et al. 1984). The staining pattern of INV has been postulated to be of aid in differential diagnosis in the distinction between benign and malignant proliferation in urothelial lesions (Walts and Said 1985) and in distinguishing cervical intraepithelial neoplasia from invasive carcinoma (Warhol et al. 1982). Lesions with marked intraepithelial inflammation revealed a decreased INV reactivity implying a modulation of INV expression by microenvironment and cellular pathological alterations (Warhol et al. 1982).

Normal breast tissue does not react with antibodies to INV (Rice et al. 1984; Walts and Said 1985). In a study of 15 breast carcinomas, INV was detected in only a few cells in two of three carcinomas of comedo type (Walts and Said 1985). However, the meaning of the positivity of these cells was not discussed further (Walts and Said 1985). In another immunohistochemical study of breast lesions antibodies to INV were used on non-neoplastic epithelial proliferations with negative results but apparently not applied to breast carcinomas (Jarasch et al. 1988).

We now report the results of an immunohistochemical study of 166 paraffin-embedded breast carcinomas using antibodies to INV and cytokeratins (CK).

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Table 1. Involucrin (INV) reactivity in 166 cases of breast carcinomas

Histological subtype	Number of cases	INV positive	INV negative
Invasive ductal (NOS)	58	18	40
Medullary	53	17	36
Mucinous	28	2	26
Invasive lobular	11	1	10
Intraductal[a]	5	2	3
Ductal, Paget's disease[b]	3	0	3
Squamous cell	2	2	0
Ductulo-lobular	2	0	2
Apocrine	1	1	0
Adenoid-cystic	1	0	1
Tubular	1	0	1
Papillary	1	0	1
Total number	166	43 (25.9%)	123 (74.1%)

[a] Intraductal mixed, solid, comedo type
[b] Invasive ductal carcinoma with Paget's disease of the nipple

Materials and methods

One hundred and sixty-six paraffin-embedded breast carcinomas of various histological subtypes (Table 1), classified according to the criteria of WHO (1981), were studied for the presence of INV-positive tumour cells. The specimens, selected retrospectively, were obtained from 162 females and 4 males. Immediately after surgical removal all specimens were fixed in 10% phosphate-buffered formaldehyde solution (pH 7.4), embedded in paraffin, and processed conventionally. Consecutive sections, 5 µm thick, were stained with haematoxylin and eosin (H&E).

Patterns of INV positivity were compared with staining for CK using monoclonal antibodies (EAB 903, EAB 904, PKK1) in parallel sections. The antibodies used in this study are described in Table 2. In selected cases, in addition, double immunostaining was applied to demonstrate coexpression of INV and CK.

For comparison of immunohistochemical staining patterns and for control purposes, normal squamous epithelium from breast and oesophagus, and squamous cell carcinomas of the breast and one oesophageal carcinoma were also investigated with antibodies to high molecular weight CK (EAB 903, EAB 904) and INV.

For demonstration of CK the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method was used (Cordell et al. 1984). The presence of INV was demonstrated by peroxidase-anti-peroxidase staining (Said et al. 1983).

For CK staining, the sections were pretreated with 0.1% protease (type XIV; Sigma, St. Louis, USA.) for 30 min at room temperature. Double immunostaining employing these methods was performed to recognize coexpression of CK and INV. The peroxidase enzyme reaction was developed using 0.02% 3.3′-diaminobenzidine tetrahydrochloride yielding a brown colour. In the APAAP method the enzyme reaction was developed with a fast red/naphthol/-As-Bi solution (diluted in a veronal-acetate buffer with the addition of 100 mg levamisol [Sigma] per 50 ml of the mixture) for 45 min at room temperature. The result was a red staining reaction.

For control purposes, tissues known to contain the respective antigens were included (positive controls). Replacement of the primary antibody by normal serum revealed negative results (negative controls).

Results

Normal breast tissue present around carcinomatous areas remained unstained for INV. In specimens of the breast, normal epidermis and normal oesophagus, approximately the upper third of squamous epithelium stained for INV intracytoplasmically, whereas basal keratinocytes and immediately suprabasally situated layers of the stratum spinosum were unstained.

The suprabasal layers of the mamillary epithelium showed cytoplasmic reactivity with EAB 904, whereas EAB 903 revealed full-thickness staining of squamous epithelium. A similar reactivity with EAB 903 was seen in oesophageal epithelium, whereas EAB 904 revealed negative results. In oesophageal squamous cell carcinoma only a few scattered tumour cells were decorated by antibodies to INV and by EAB 903, whereas no positivity could be detected with EAB 904. Reactivity in tumour cells of squamous cell breast carcinoma with INV, EAB 903 and EAB 904 antibodies was similar to that of oesophageal carcinoma.

In this study 43 of 166 breast carcinomas (25.9%) of various histological subtypes showed INV-positive neoplastic cells (Table 1).

Eighteen of 58 invasive ductal carcinomas NOS (31%) contained INV-positive tumour cells. The staining was moderate to strong and predominantly diffuse within the cytoplasm (Fig. 1A). In addition, in three cases some tumour cells revealed a strong accentuation of staining along the cell membranes and around the nucleus (Fig. 1B). In four cases squamous cell-like differentiation with homogeneous eosinophilic cytoplasm could be seen in H&E-stained sections; however, complete squamous cell metaplasia with intercellular bridges or keratinization was not present. These cells showed a strong diffuse cytoplasmic INV reactivity. The number of INV-positive tumour cells varied greatly from case to case. In eight cases only single cells or few small cell clusters were

Table 2. List of antibodies used for immunohistochemistry

Antibody	Donor	Molecular weights ($\times 10^{-3}$) of CK recognized	Dilution	Source
CK EAB 904	Mouse, monoclonal	57, 66	1:20	1
CK EAB 903	Mouse, monoclonal	57, 66	1:5	1
CK PKK1	Mouse, monoclonal	44, 46, 52, 54	1:200	2
Involucrin	Rabbit, polyclonal		1:10	3

Sources: 1, Enzo Diagnostics, New York, USA; 2, Labsystem Oy, Helsinki, Finland; 3, Biomedical Technologies, Stoughton, Mass., USA

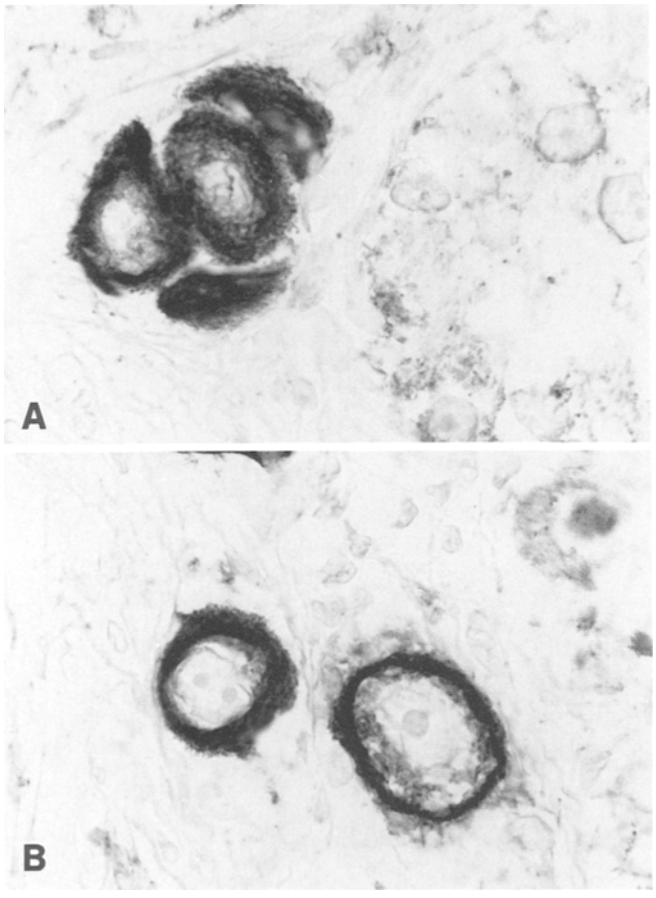


Fig. 1A, B. Involucrin positivity in tumour cells of invasive ductal carcinoma with (**A**) diffuse cytoplasmic reactivity and (**B**) accentuated staining along the cell membrane and around the nucleus. Peroxidase–anti-peroxidase, ×480

INV-positive (Fig. 2A). The remaining ten cases contained a high number of INV-positive tumour cells (up to 70% of tumour cells) showing either a homogeneous cytoplasmic staining of large tumour cell groups (Fig. 2B) or patchy reactivity of many tumour cells resembling a checkerboard of stained and unstained cells (Fig. 2C). In some cases, INV-positive tumour cells were arranged at the periphery of INV-negative carcinoma cells, revealing a rim of stained around unstained cells (Fig. 2D).

In cases with INV-positive tumour cells parallel sections were studied for cytokeratin expression. Using EAB 904, in 9 of 18 cases positive tumour cells were observed. The number of immunoreactive cells was variable in each case. Double immunostaining using EAB 904 and INV antibodies (two cases) revealed coexpression in only a small number of tumour cells (Fig. 3).

In 2 of 18 cases a very weak cytoplasmic reactivity of a few dispersed tumour cells was detected with EAB 903. No coexpression with INV was found.

Most tumour cells were strongly stained with PKK1. Coexpression of PKK1 and anti-INV staining could be observed in almost all INV-positive tumour cells.

Seventeen of 53 medullary carcinomas (32%) contained INV-positive tumour cells. In 6 of 17 cases squamous-like differentiation of some tumour cells was seen in H&E stained sections, although true squamous metaplasia showing horny pearls or intercellular bridges was not observed. These cells were strongly INV positive (Fig. 4). The number of INV-positive tumour cells varied considerably. In 11 carcinomas a large number of tumour cells (up to 60% of total cell number) was stained for INV, the positive cells being either arranged in large groups or interspersed among nonreactive cells in a mosaic pattern.

In parallel sections, a variable number of EAB 904-positive tumour cells was found in 15 carcinomas. Three cases, which showed a similar distribution pattern of anti-INV- and EAB 904-positive cells, were studied by double immunostaining. Coexpression was found in some, but not all tumour cells.

Tumour cells were nonreactive with EAB 903. Most carcinoma cells were PKK1 positive, and coexpression of CK revealed by PKK1 and INV was seen in almost all tumour cells.

In two mucinous carcinomas (7.1%), a few scattered tumour cells, not showing squamous differentiation had weak cytoplasmic INV reactivity. The tumour cells remained unstained with antibodies EAB 903 and EAB 904. Most tumour cells were, however, strongly reactive with PKK1.

In the one case of invasive lobular carcinoma a weak cytoplasmic INV reactivity was seen in about 20% of the dispersed tumour cells. With EAB 904 a weak cytoplasmic reactivity was observed in about 20% of carcinoma cells, the distribution pattern of reactive cells being similar to those with INV reactivity. None of the tumour cells were EAB 903 positive, but most were PKK1 positive. Due to lack of material no double immunostaining could be performed.

In the intraductal carcinomas, of mixed, solid and comedo type two cases (mixed type with comedo pattern predominating and solid type) showed many INV-positive tumour cells. The reactive cells were found in solidly arranged areas, showing weak-moderate diffuse cytoplasmic reactivity (Fig. 5A). Occasionally, INV-positive tumour cells were located in peripheral areas surrounding mostly INV-negative carcinoma cells. No reactivity was detected with EAB 904. Using EAB 903 a strong cytoplasmic positivity was seen in myoepithelial cells, whereas the tumour cells, many of them INV positive, failed to react (Fig. 5B). Most tumour cells were PKK1 positive, mostly with coexpression of CK and INV.

We also examined uncommon types of invasive carcinomas (ductal carcinoma with Paget's disease of the nipple; ductulo-lobular; adenoid-cystic; tubular; papillary). The tumour cells in ductal carcinomas with Paget's disease of the nipple did not stain positively for INV, whereas INV reactivity could be seen in keratinocytes. The tumour cells in the remaining histological subtypes failed to react with INV. CK staining was not performed in these cases.

The tumour cells in squamous cell carcinomas showed a strong cytoplasmic reactivity of a few scattered cells with anti-INV and EAB 903. INV was present in the

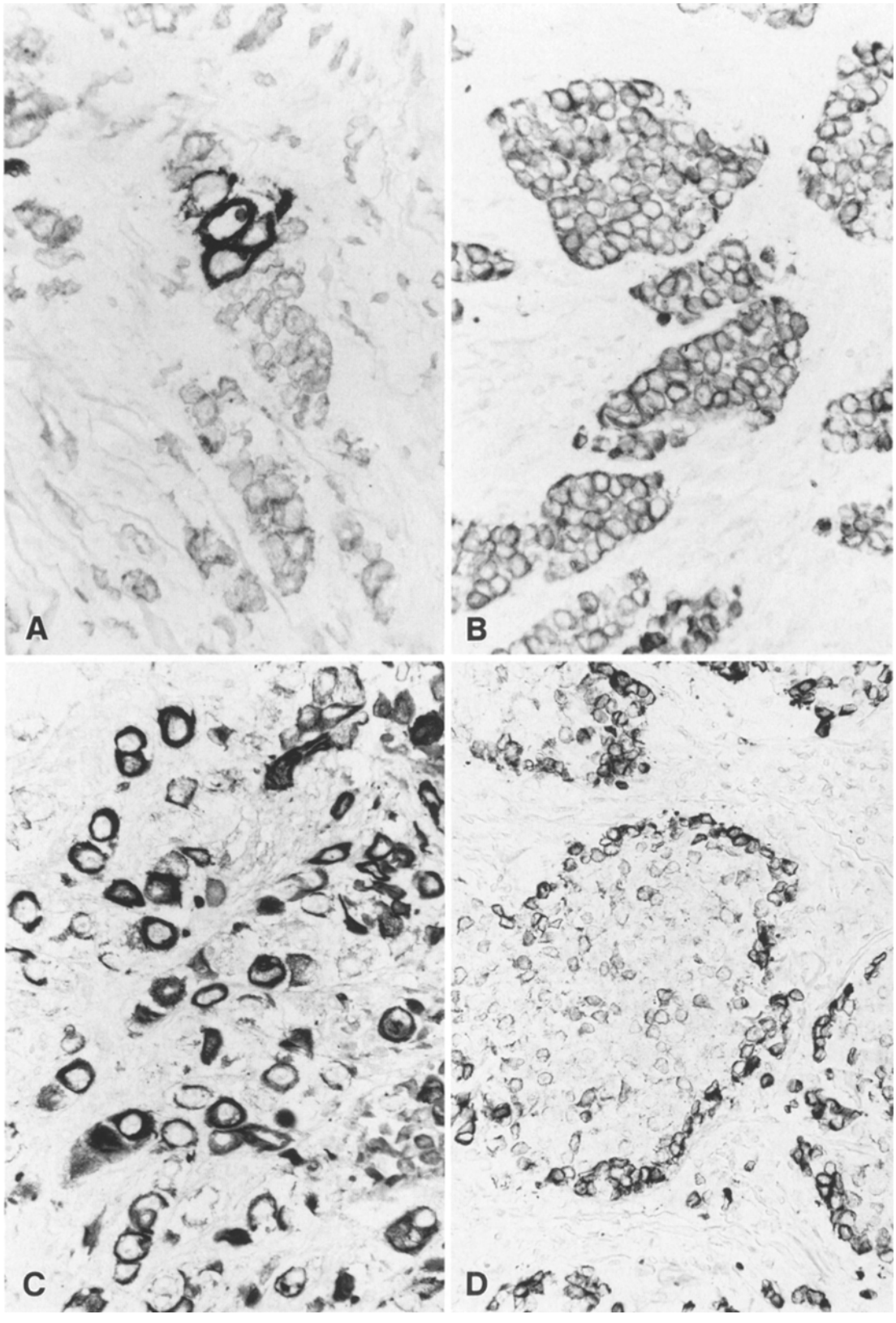
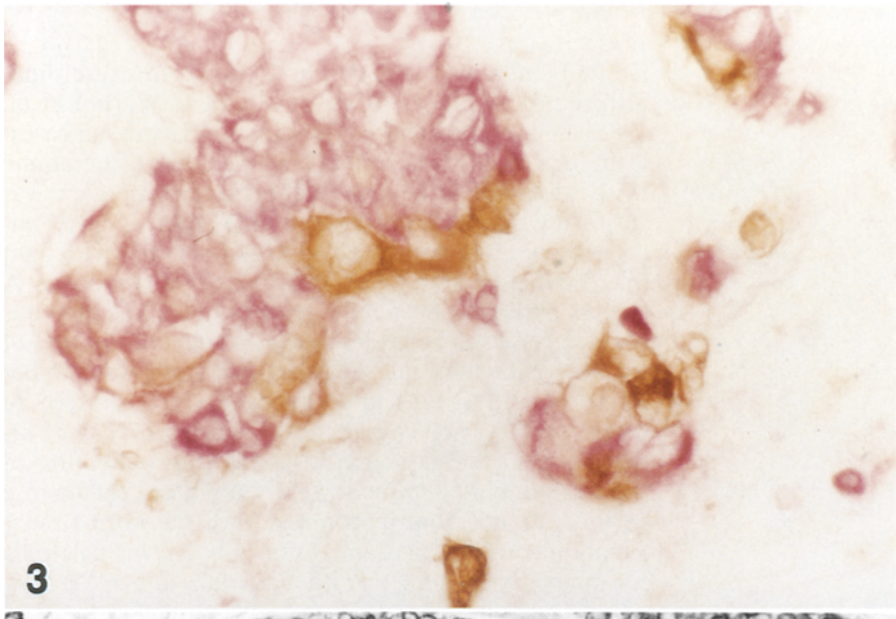
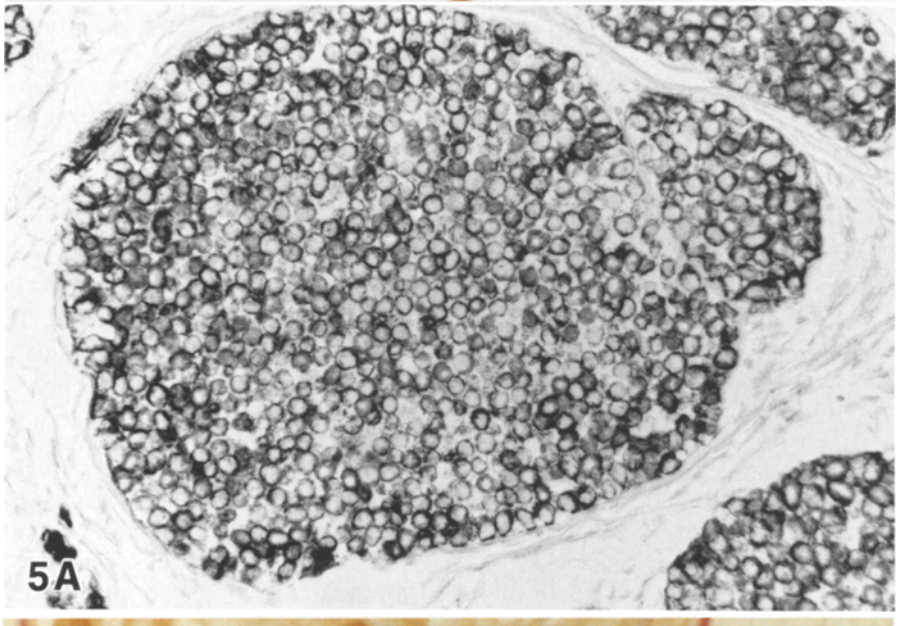


Fig. 2A–D. Varying number of involucrin (INV)-positive tumour cells with variable staining patterns in invasive ductal carcinomas. **A** Few immunoreactive cells, **B** immunoreactivity of large tumour cell groups, **C** checkerboard pattern of reactive and non-reactive tumour cells, **D** immunoreactive tumour cells in the periphery of INV-negative carcinoma cell groups. Peroxidase–anti-peroxidase, × 320





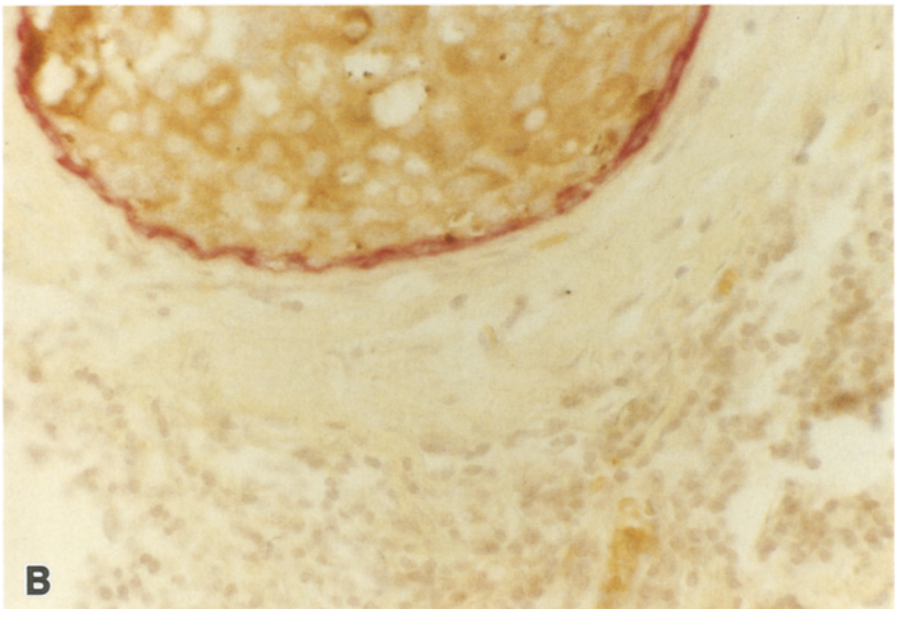


Fig. 3. Coexpression of involucrin *(brown)* and cytokeratin EAB 904-staining *(red)* in a few tumour cells of invasive ductal carcinoma. Double immunostaining, × 350

Fig. 5. Involucrin (INV) positivity in intraductal carcinoma showing reactivity (**A**) of many tumour cells. **B** Myoepithelial cells are EAB 903-positive (red colour), whereas INV-positive tumour cells (brown colour) lack reactivity with EAB 903. **A** Peroxidase–anti-peroxidase, × 340; **B** double immunostaining, × 380

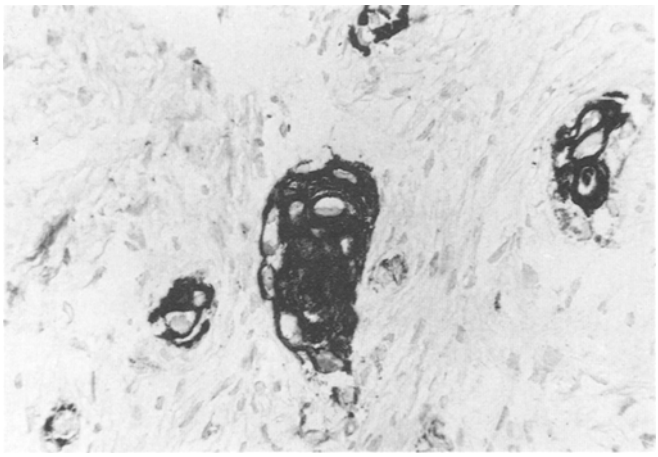


Fig. 4. Involucrin reactivity in cells with squamous-like differentiation in medullary carcinoma. Peroxidase–anti-peroxidase, ×360

suprabasal layers of the overlaying skin. No reactivity of tumour cells was seen with EAB 904. Double stainings and stainings using PKK1 were not performed due to lack of material.

A few scattered tumour cells in an apocrine corcinoma showed cytoplasmic INV reactivity. No reactivity was seen using the antibody EAB 904. Due to lack of material no further studies could be performed.

Discussion

Involucrin, a nonfilamentous cytoplasmic protein synthesized during squamous differentiation, is known to be a distinctive marker for near terminal differentiation of keratinocytes of stratified squamous epithelium in the skin, oral cavity, ectocervix, vagina and oesophagus (Rice et al. 1984; Kamino et al. 1988). INV has been shown to be immunologically and biochemically unrelated to CK (Rice and Green 1979; Sun and Green 1976; Green 1979). Previous immunohistochemical studies demonstrated INV positivity in various tumour cells with squamous differentiation (Said et al. 1983; Watt and Phil 1983; Rice et al. 1984; Murphy et al. 1984; Elsayed et al. 1987; Klein-Szanto et al. 1987; Kamino et al. 1988). Whereas normal breast tissue and invasive ductal and medullary breast carcinomas were shown to be negative for INV by Rice et al. (1984) and Walts and Said (1985), Walts and Said (1985) reported weak INV positivity in two of three comedocarcinomas of the breast. In their study, however, the reactive cells in these tumours were not defined further.

In the present study, we evaluated the distribution of INV in a large number of paraffin-embedded breast carcinomas of various histological subtypes and, in selected cases, related INV positivity to CK reactivity. In blots, both EAB 903 and EAB 904 identify high molecular weight keratins of 66 kDa and 57 kDa. However, in tissues different reactivities of these antibodies are well known (Gown and Vogel 1982, 1984). INV-positive tumour cells were detected in 43 of 166 breast carcinomas (25.9%). Inflammation, which is known to cause diminished INV synthesis (Rice et al. 1984; Warhol et al. 1985) did not apparently influence reactivity in our cases; a finding particularly evident in medullary carcinoma. With the exception of squamous breast carcinoma, it was surprising that INV positivity in the remaining histological subtypes was not restricted to tumour cells with light microscopic phenotype of squamous differentiation. A similar observation of INV reactivity in non-squamous tumour cells has been described in adenocarcinoma of the lung (Mayall et al. 1992). In our study the number of INV-positive cells and the distribution pattern of reactive cells varied considerably from case to case. Furthermore, under pathological conditions, activation of the cross-linking enzyme (transglutaminase) occurs in a perinuclear position, in contrast with the normal submembraneous localization. No or only a few single cells coexpress INV and CK with EAB 903 and EAB 904, reflecting the well known heterogeneity of CK (Moll et al. 1982) and a possible dissociation in the regulation of INV and CK. In mammary squamous cell carcinomas, INV was present in only a few scattered tumour cells, a finding which is comparable with INV reactivity in squamous cell carcinoma of the lung (Mayall et al. 1992). This is in contrast with INV staining in squamous cell carcinoma of the skin, where increased INV staining has been reported (Said et al. 1984).

One possible explanation of our findings is that INV positivity of tumour cells might indicate premature or abnormal squamous cell differentiation, which cannot be recognized by light microscopy. However, an unidentified protein that cross-reacts with INV cannot be excluded at the present time.

The discrepancies between previous studies on breast carcinoma studies using INV antibodies (Rice et al. 1984; Walts and Said 1985) and our data might be due to the smaller number of cases studied previously and to variability of INV expression in tumour cells. Increased sensitivity of the techniques employed may also be relevant.

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