

A useful immunohistochemical approach to evaluate intraductal proliferative lesions of the breast and to predict their prognosis

Yoko Omi¹, Tomoko Yamamoto^{1,3}, Takahiro Okamoto², Takao Obara² and Makio Kobayashi^{1,3}

Departments of ¹Pathology, ²Endocrine Surgery and ³Surgical Pathology, Tokyo Women's Medical University, Shinjuku-ku, Tokyo, Japan

Summary. An examination was performed on 16 intraductal proliferative breast lesions diagnosed as intraductal papillomas (IP) or usual ductal hyperplasia (UDH), which were followed up for more than 3 years. An immunohistochemical marker panel combining myoepithelial markers, high-molecular-weight keratin (HMWK) and neuroendocrine markers was used. Two of 11 IP cases were re-evaluated as atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS). These cases developed breast cancer after the first operation. One IP case showed repeated recurrences. None of the other IP and UDH cases had breast cancer or recurrence. The ADH, DCIS and the recurrent IP showing a solid growth lacked myoepithelia, but the recurrent IP expressed HMWK, immunohistochemically. Interestingly, these three lesions were weakly positive for neuroendocrine markers. All other IPs and UDHs, including lesions having solid components, were negative for neuroendocrine markers, and most of them were positive for myoepithelial markers and/or HMWK. A combination of the above immunohistochemical markers seems useful to evaluate intraductal proliferative lesions and to predict their prognosis. In particular, intraductal proliferative lesions with solid components exhibiting positivity for neuroendocrine markers should be followed up carefully to monitor breast cancer risk or recurrence.

Key words: Immunohistochemistry, Intraductal proliferative lesions, Prognosis

Introduction

Immunohistochemistry is a widely available technique that assists the pathological diagnosis of intraductal breast lesions. Several antibodies are generally used to distinguish benign intraductal proliferative lesions from ductal carcinoma *in situ* (DCIS). It is well known that myoepithelial cells are absent or reduced in DCIS (Papotti et al., 1983; Raju et al., 1989; Hill and Yeh, 2005). p63, calponin, smooth muscle actin, CD10 (Tse et al., 2007) and smooth muscle myosin are markers for staining myoepithelial cells. In addition to these myoepithelial markers, high-molecular-weight keratin (HMWK) is a good tool for differentiating malignancy in solid-growing epithelial cells. Usual ductal hyperplasia (UDH) and intraductal papilloma (IP) show an intense mosaic staining of HMWK, in contrast to the lack of immunoreactivity in DCIS (Tse et al., 2007). Cytokeratin 34BE12 (CK34BE12), CK 5/6 and CK14 are routinely available HMWK (Tse et al., 2007). Stainings of neuroendocrine markers, such as chromogranin A and synaptophysin, are reported to show a positive reaction in some solid intraductal papillary carcinomas (Tse et al., 2007). Moritani et al. (2007) proposed an immunohistochemical panel to distinguish DCIS and IP by combining these markers.

Most of the patients with benign disease think that they are cured after its removal and tend to stop attending follow-ups. However, proliferative breast diseases such as IP, adenosis, UDH, atypical ductal hyperplasia (ADH) or atypical lobular hyperplasia are known to be risk factors for breast cancer (Dupont and Page, 1985; Carter et al., 1988; London et al., 1992; Bodian et al., 1993; Schnitt, 2003; Hartmann et al., 2005; Worsham et al., 2009). It is a matter of great interest for breast surgeons to determine the significant

risk factors for breast cancer among patients with proliferative breast disease and to alert patients that need careful follow-up. Previous studies have suggested that the relative risk of breast cancer is increased in proliferative lesions with atypia compared with that of proliferative lesions without atypia as defined by a morphological approach (Dupont and Page, 1985; Carter et al., 1988; London et al., 1992; Bodian et al., 1993; Schnitt, 2003; Hartmann et al., 2005; Worsham et al., 2009). However, there have been no immunohistochemical studies that assessed the breast cancer risk in patients with benign intraductal proliferative disease, to the authors' knowledge. In this study, using the immunohistochemical panel proposed by Moritani et al. (2007), we re-evaluated intraductal proliferative lesions that were diagnosed as benign, and investigated whether or not the panel is effective for predicting prognosis.

Materials and methods

Patients

Sixteen surgically resected specimens of 15 patients were selected from the case file of the Department of Surgical Pathology of Tokyo Women's Medical University Hospital from 1995 to 2003. The operations were performed at the Department of Endocrine Surgery, Tokyo Women's Medical University Hospital. Patients followed up for at least 3 years were considered to be eligible for this study. Cases containing carcinoma in the specimens resected at diagnosis were excluded. The lesions were 11 IPs and 5 UDHs at diagnosis. This study was approved by the Ethical Committee of Tokyo Women's Medical University.

Histological evaluation

Two investigators (Y.O. and T.Y.) reviewed the original hematoxylin and eosin (H & E) specimens of all cases, according to the WHO classification (MacGrogan et al., 2003a; Tavassoli et al., 2003). IP was defined as a proliferation of epithelial and myoepithelial cells overlying fibrovascular stalks creating an arborescent structure within the lumen of a duct. UDH was defined as a benign ductal proliferative lesion typically characterized by secondary lumens, and streaming of the central proliferating cells.

Immunohistochemistry

The primary antibodies employed in immunohistochemistry were mouse monoclonal IgGs against p63 (Clone: 4A4, 1:50, Dako, Glostrup, Denmark), smooth muscle myosin (Clone: SMMS-1, 1:100, Dako), CK34βE12 (Clone: 34βE12, 1:100, Dako), CK 5/6 (Clone: D5/16B4, diluted 1:50, Dako), synaptophysin (Clone: SY38, 1:100, Dako) and chromogranin A (Clone: M869, 1:100, Dako).

Three-μm-thick sections of each material were cut

from a representative tissue block of each case. Sections were deparaffinized, rehydrated, quenched for 5 min at room temperature with 3% H₂O₂, and rinsed in phosphate-buffered saline, pH 7.6 (PBS). The sections were processed with microwaving: 95°C, 400 W, 20 min in 10 mM citrate buffer (pH 6.0) for smooth muscle myosin and synaptophysin, 95°C, 400 W, 40 min in 10 mM citrate buffer (pH 6.0) for chromogranin A, and 95°C, 400 W, 40 min in 1 mM Tris-EDTA (pH 9.0) for p63 and CK 5/6. A treatment with protease for 10 min at room temperature was employed for CK34βE12. After a pretreatment with 3% non-immune animal serum in PBS for 30 min at room temperature, the sections were incubated overnight at 4°C with the primary antibodies. Antibody binding was visualized by the avidin-biotin-immunoperoxidase complex method using Vectastain ABC kits (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. 3,3'-diaminobenzidine tetrahydrochloride was used for the chromogen and hematoxylin for the counterstain.

For evaluation of immunohistochemistry, definitions were set according to the criteria proposed by Moritani et al. (2007). For the myoepithelial markers, the cells at the epithelial-stromal interface of the intraluminal proliferating component were evaluated. Positive cells outlining the duct were excluded from evaluation. For HMWK and neuroendocrine markers, intraluminal proliferating cells were examined. The intensity of immunoreaction was divided into negative, weakly positive and positive. For myoepithelial markers and HMWK, intensity of immunoreaction was compared with that of myoepithelia and luminal cells of the normal duct. For neuroendocrine markers, neuroendocrine-marker-positive DCIS was used as a positive control. When immunoreactive cells were comprised less than 10% of the lesion, it was determined to be negative. It was considered to be weakly positive when more than 10% of cells were stained, but the intensity was weaker than that of controls, and was considered to be positive when more than 10% of cells were stained at the same or a stronger intensity than the controls.

Results

Clinicopathological findings

The clinicopathological findings are summarized in Tables 1 and 2. The mean ages of the patients with IP and UDH were 46.2 (range, 28-69) and 50.6 years (range, 39-64), respectively. One patient with IP was male and the rest of the patients were female. 10 IP and 5 UDH patients were followed for medians of 8.2 (3.2-14.3) and 9.4 years (5.1-12.3), respectively.

All of the IPs were single. One patient with IP in the right breast exhibited recurrence of IP in the left breast after three years (Case 1). This patient had experienced resections of IP in the bilateral breast, 20 years and 27 years before, but the specimens could not be obtained for this study. A solid growth pattern was seen in the

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recurred lesion (Fig. 1). Two lesions originally diagnosed as IP revealed cellular atypia (Cases 2 and 3). In Case 2, a lesion showing solid or cribriform intraductal architecture measuring less than 2 mm was considered to be ADH after the review (Fig. 2). The patient developed invasive ductal carcinoma at the

operative scar 2.6 years after the operation. Case 3 was re-evaluated to be DCIS because atypical cells showed monotonous proliferation with solid or cribriform structure (Fig. 2). In this patient, DCIS occurred at the operative scar 4 years and 5 months later.

Among the rest of the IP cases, Cases 6-8 and 10 had

Table 1. Summary of 11 intraductal papillomas.

Case	Age	Sex	Immunohistochemistry						Solid growing component	Breast cancer occurrence (Period after surgery)
			SMM	p63	34βE12	CK 5/6	Syn	CA		
1 (primary)	46	F	++	++	++	++	-	-	Absent	No
(recurrence)	50	F	-	-	++	++	+	-	Present	No
2	38	F	-	-	-	-	+	+	Present	Yes (2 years and 8 months)
3	39	F	-	-	-	-	+	-	Present	Yes (4 years and 5 months)
4	57	M	-	-	-	-	-	-	Absent	No
5	69	F	++	++	++	++	-	-	Absent	No
6	46	F	++	++	++	-	-	-	Present	No
7	50	F	++	++	++	++	-	-	Present	No
8	38	F	++	++	++	++	-	-	Present	No
9	51	F	++	++	++	++	-	-	Absent	No
10	28	F	++	++	++	++	-	-	Present	No

F: Female; M: Male; SMM: Smooth muscle myosin; CK: Cytokeratin; Syn: Synaptophysin; CA: Chromogranin A; -: Negative; +: Weakly positive; ++: Positive

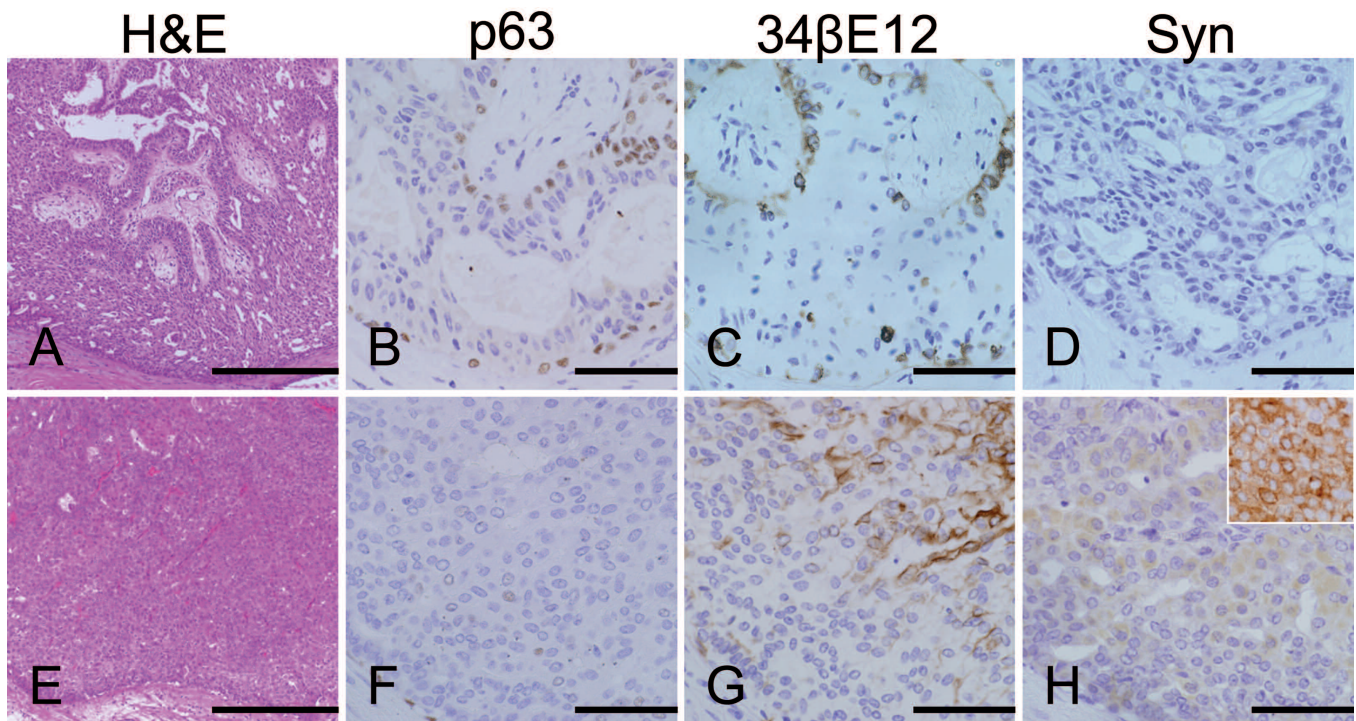


Fig. 1. Photomicrographs of Case 1 (**A and E**) and immunostaining of p63 (**B and F**), CK34βE12 (**C and G**) and Syn (**D and H**). **A-D** are primary IP and **E-H** are recurrent IP. The primary IP does not contain a solid component (**A**). p63 (**B**) and CK34βE12 (**C**) are positive and Syn is negative. The recurrent IP (**E**) consisting of a solid component is p63 negative (**F**), but CK34βE12 is maintained (**C and G**). Syn is weakly positive (**H**). The inset in **H** is the result in neuroendocrine DCIS used as a positive control. Syn: synaptophysin; IP: intraductal papilloma. Scale bars: A, E, 500 μm; B-D, F-H, 200 μm.

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Table 2. Summary of 5 usual ductal hyperplasia.

Case	Age	Sex	Immunohistochemistry						Solid growing component	Breast cancer occurrence
			SMM	p63	34 β E12	CK 5/6	Syn	CA		
11	64	F	-	-	++	++	-	-	Present	No
12	45	F	++	++	-	-	-	-	Absent	No
13	53	F	++	++	++	++	-	-	Absent	No
14	52	F	-	-	++	++	-	-	Present	No
15	39	F	++	++	-	-	-	-	Absent	No

F: Female; M: Male; SMM: Smooth muscle myosin; CK: Cytokeratin; Syn: Synaptophysin; CA: Chromogranin A; -: Negative; +: Weakly positive; ++: Positive

Table 3. Comparison between prognosis and positive immunoreactions for each marker.

	SMM	p63	34 β E12	CK 5/6	Syn	CA
IP	6/10 (60%)	6/10 (60%)	7/10 (70%)	6/10 (60%)	3/10 (30%)	1/10 (10%)
IP recurrence*	0/1 (0%)*	0/1 (0%)*	1/1 (100%)*	1/1 (100%)*	1/1 (100%)*	0/1 (0%)*
Breast cancer occurrence	0/2 (0%)	0/2 (0%)	0/2 (0%)	0/2 (0%)	2/2 (100%)	1/2 (50%)
Neither	6/7 (86%)	6/7 (86%)	6/7 (86%)	5/7 (70%)	0/7 (0%)	0/7 (0%)
UDH	3/5 (60%)	3/5 (60%)	3/5 (60%)	3/5 (60%)	0/5 (0%)	0/5 (0%)

SMM: Smooth muscle myosin; CK: Cytokeratin; Syn: Synaptophysin; CA: Chromogranin A; *: The result of the primary IP of Case 1 is excluded in this table

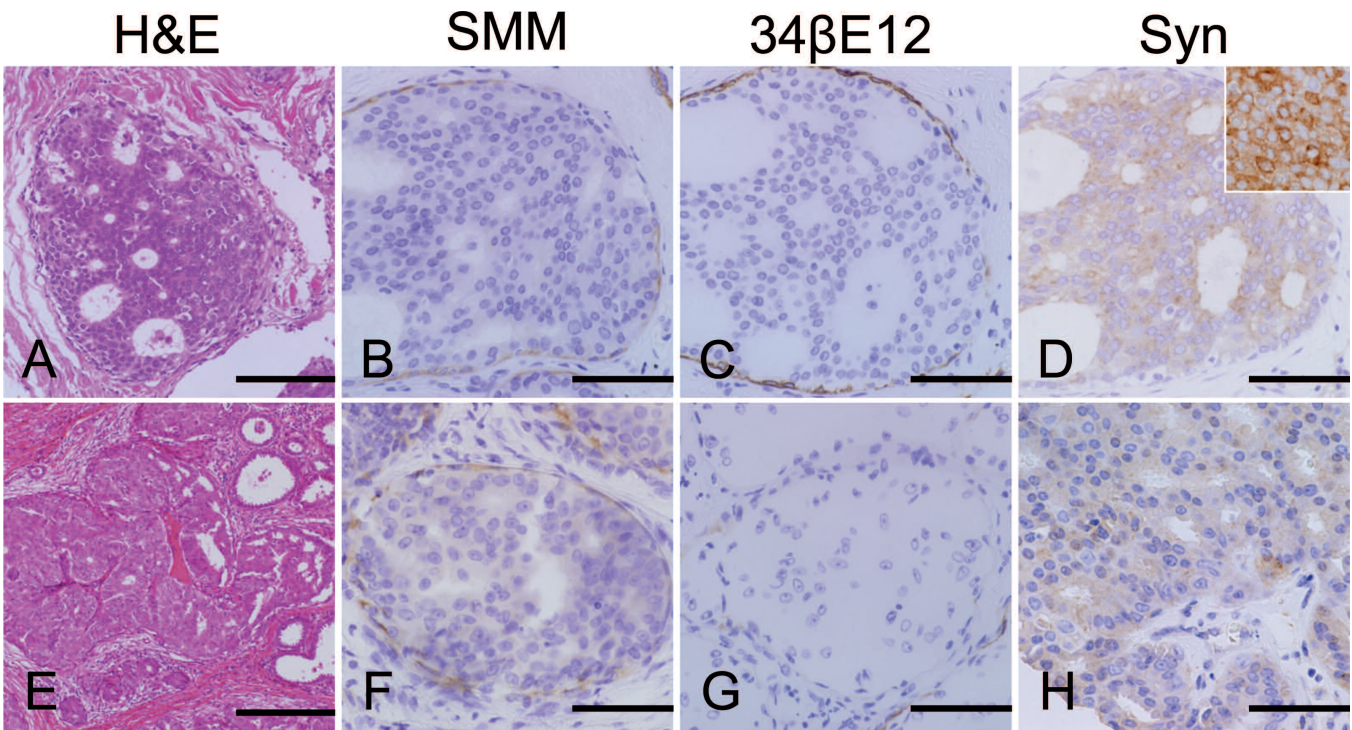


Fig. 2. Photomicrographs of Case 2 (A-D) and Case 3 (E-H). Immunostaining of SMM (B and F), CK34 β E12 (C and G) and Syn (D and H). In both cases, lesions consisting of a solid component (A and E) are SMM negative (B and F), CK34 β E12 negative (C and G) and Syn weakly positive (D and H). The inset in D shows the result in neuroendocrine DCIS used for a positive control. SMM: smooth muscle myosin; Syn: synaptophysin. Scale bars: A, E, 500 μ m; B-D, F-H, 100 μ m.

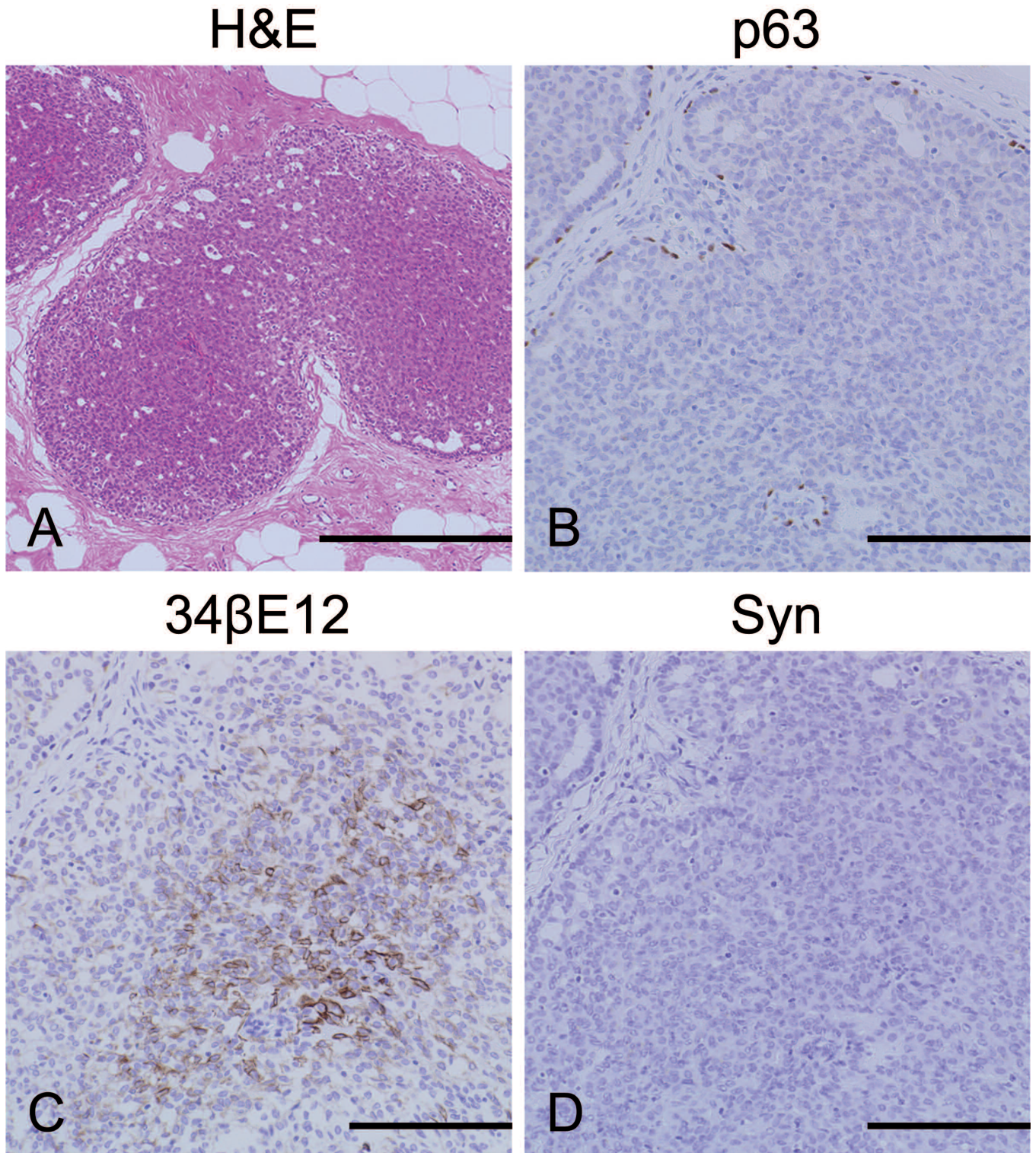


Fig. 3. Photomicrographs of Case 14. H & E staining (A), and immunostaining for p63 (B), CK34βE12 (C) and Syn (D). The lesion has a solid component (A), in which p63 is negative (B), but CK34βE12 is positive (C) and Syn is negative (D). Syn: synaptophysin. Scale bars: A, 500 μ m; B-D, 100 μ m.

solid-growing components. UDH was seen in a part of mastopathy. Two UDH patients (Cases 11 and 14) showed solid growth of duct epithelia (Fig. 3). Seven IP and 5 UDH patients did not recur or develop malignancy.

Immunohistochemistry

In Case 1, immunoreactivity against HMWK was maintained in both primary and recurrent IPs, but myoepithelial cells were absent. Synaptophysin was expressed in the recurrent IP, although the intensity was weak (Fig. 1). In Case 2, myoepithelial markers and HMWK were negative, and neuroendocrine markers were weakly positive (Fig. 2). In Case 3, myoepithelial markers and HMWK were absent, but for neuroendocrine markers, only synaptophysin was faintly stained (Fig. 2). In other cases with solid components, myoepithelial markers and/or HMWK were positive and neuroendocrine markers were negative (Fig. 3)

The immunohistochemical findings are summarized in Tables 1-3. In Table 3, the primary lesion of Case 1 is conventionally omitted. Myoepithelial markers were positive in 6 of 10 IP cases and 3 of 5 UDH cases. None of these cases recurred or developed breast cancer. There was no discrepancy in the results of smooth muscle myosin and p63. Among 6 myoepithelial-marker-negative cases, one was a case with recurrent IP (Case 1). Two cases developed subsequent breast cancer (Cases 2 and 3) and 3 other cases (Cases 4, 11, 14) did not recur or develop cancer.

CK34BE12 was positive in 7 of 10 IP and 3 of 5 UDH cases. CK5/6 was positive in 6 of 10 IP cases and 3 of 5 UDH cases. HMWK-positive cases did not show recurrence or subsequent cancer development except for one case (Case 1), while two HMWK-negative cases developed breast cancer (Cases 2 and 3).

Synaptophysin was weakly positive in three lesions: one recurrent IP (Case 1) and two lesions re-evaluated as ADH and DCIS (Cases 2 and 3). Chromogranin A was weakly positive only in Case 2. All other cases were negative for neuroendocrine markers and did not recur or develop cancer.

Discussion

For the differential diagnosis to evaluate malignant potential in intraductal proliferative lesions, evaluation of solid components is one of the most important and difficult issues. Immunohistochemistry is used to support observations in H&E staining. Moritani et al. (2007) reported that solid intraductal lesions that meet at least two of the following criteria are likely to be malignant: 1) absence of myoepithelial cells, 2) negativity for HMWK and 3) positivity for neuroendocrine markers.

The most fundamental feature of benignity is the preservation of myoepithelial cells along the epithelial-stromal interface. However, the myoepithelium is

sometimes difficult to distinguish from the ductal epithelium in H&E staining. p63 shows nuclear staining and is reported to have the highest sensitivity among myoepithelial markers (Tse et al., 2007). We used smooth muscle myosin that stains the cytoplasm in addition to p63, but the immunohistochemistry for myoepithelial cells is not always effective. In IP and UDH with solid components in particular, myoepithelial cells compressed to the periphery of the duct sometimes make it difficult to differentiate the lesion from DCIS, even if immunohistochemistry is applied. In fact, Case 1 (IP with recurrence), Case 11 and Case 14 (UDH) containing solid components within the ducts were negative for myoepithelial markers.

To solve this problem, immunohistochemistry for HMWK, which stains basal cells and sometimes myoepithelial cells, is known to be useful (Boecker et al. 2001; Moriya et al., 2006). CK34BE12 is a cocktail of CK1, 5, 10 and 14. In distinguishing benign from malignant cases, Tan et al. (2005) showed higher sensitivity and specificity in CK 5/6 and CK14 staining than in CK34BE12. In IP and UDH, basal cells proliferate heterogeneously with luminal cells, which results in mosaic immunostaining of HMWK (Tse et al., 2007). In contrast, carcinoma is a monotonous tumor cell growth. Indeed, solid components in Case 1, 11 and 14 were negative for myoepithelial markers, but showed a mosaic staining of HMWK. In contrast, solid components of Case 2 (ADH) and Case 3 (DCIS) were negative for both myoepithelial markers and HMWK.

Some DCIS having solid sheet-like growth transversed by fibrovascular septa exhibit neuroendocrine differentiation (Cross et al., 1985; Maluf and Koerner, 1995). In this neuroendocrine DCIS, intraductal cells do not appear to be monotonous and the nuclear grade (The Consensus Conference Committee, 1997) is low (Kawasaki et al., 2008), leading to misdiagnosis as IP. Thus, the use of neuroendocrine markers is reported to be useful, presenting with a higher positivity in synaptophysin than in chromogranin A (Tsang and Chan, 1996). In our study, chromogranin A was positive in one ADH patient. Synaptophysin was weakly positive in one ADH and one DCIS case, although their staining intensities were fainter than that of typical neuroendocrine DCIS.

The immunohistochemical panel used in this study was found to be a useful tool to re-evaluate intraductal proliferative lesions, and may be applicable for further retrospective studies in past cases that have been diagnosed only by H&E staining, particularly in cases showing solid growth. In most of the literature, mainly in older reports, the definition of ADH and DCIS is unclear and the diagnosis is made by H&E staining. Thus, cases diagnosed as benign, particularly cases developing subsequent carcinoma, might include cases that should be classified as ADH or DCIS, as in our study, if sufficient immunohistochemical analysis was performed under the criteria of the WHO classification (MacGrogan et al., 2003a; Tavassoli et al., 2003).

Immunohistochemical analysis of intraductal proliferative lesions of the breast

It has already been reported that proliferative breast disease has a higher risk of causing breast cancer than non-proliferative disease (Dupont and Page, 1985; Carter et al., 1988; London et al., 1992; Bodian et al., 1993; Schnitt, 2003; Hartmann et al., 2005; Worsham et al., 2009). The risk becomes even higher when cellular atypia is exhibited. According to the studies of Lewis et al. (2006) and Worsham et al. (2007), breast cancer risk is greater in multiple benign breast disease. In a study of 119 IPs, 5 recurrences comprising papillomas, 2 carcinomas arising in papilloma, 4 DCISs and 5 invasive ductal carcinomas were observed during follow-up (MacGrogan and Tavassoli, 2003b). Proliferative breast disease in the surrounding tissue and infarction of the IP were predictive factors of recurrence. There were neither IP with infarction nor multiple papillomas in our study.

The immunohistochemical panel used in this study also appears to be helpful to predict the prognosis of intraductal proliferative lesions in addition to re-evaluation. Among them, neuroendocrine markers are intriguing. In the present study, most of the cases that did not develop breast cancer were positive for at least either myoepithelial markers or HMWK, but there was one case negative for both neuroendocrine markers and HMWK. On the other hand, neuroendocrine markers were only positive in cases with subsequent breast cancer or with recurrence. The most interesting result is that the recurrent IP was weakly positive for synaptophysin. In predicting the prognosis of IP and UDH, the immunohistochemistry of neuroendocrine markers appears to be a candidate for more accurate prediction of the prognosis, although further accumulation of cases is necessary. It may be difficult to predict the recurrence on the primary IP in cases such as Case 1, which was negative for neuroendocrine markers. However, the recurrent IP showed immunoreactivity of synaptophysin, which might imply the acquisition of a more malignant potential.

In conclusion, intraductal proliferative lesions of the breast with a solid component that exhibit absence of myoepithelium, negative HMWK and especially positivity for neuroendocrine markers should be followed up carefully after resection to monitor of breast cancer risk or recurrence.

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