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Immunohistochemical and Fluorescence In Situ Hybridization Studies on Noninvasive and Invasive Extramammary Paget's Disease

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Abstract

To determine useful immunohistochemical markers for tumor cells in extramammary Paget's disease (EMPD), immunohistochemical (IHC) examinations in 17 patients with EMPD, including 4 patients with dermal invasion, were performed. Among the antibodies examined, cytokeratin 7 (CK7) and CK19 were strongly positive for both intraepidermal and dermally invasive tumor cells in all patients. CAM5.2 and mucin 1 (MUC1) were also good markers. Although IHC examination revealed positive for HER-2 in 4 EMPD patients with dermal invasion, 4 out of 13 noninvasive patients were IHC negative. Fluorescence in situ hybridization (FISH) study revealed negative results for *HER-2* gene amplification in 8 IHC positive patients, including each 4 patients of both noninvasive and dermal invasive cases. Our results show that besides CK7, CK19 is another favorable marker of tumor cells of EMPD. Four patients with dermal invasion were strongly positive for HER-2, although negative results were obtained in the FISH study. Further investigations are required to confirm the results of the FISH study.

Keywords

extramammary Paget's disease, immunohistochemistry, fluorescence in situ hybridization (FISH), CK19, HER-2

Introduction

Extramammary Paget's disease (EMPD) mostly involves the genital regions or scrotum. Tumor cells mainly proliferate in the epidermis, but in some patients, aggressive tumor cells invade the dermis and metastasize to the lymph nodes and some show mucus production. It is difficult to determine the tumor margins of epidermal and dermal invasion in patients with EMPD by histological examination. Markers of EMPD cells, such as cytokeratins (CK),[1-4] carcinoembryonic antigen (CEA),[1,2] epithelial membrane antigen (EMA),[1,2] and mucin 1 (MUC1)[3,5] have been reported to determine intraepidermal and dermal invasion.[6-13] Identification of specific markers of tumor cells will be extremely useful in clinical practice.

Thus, to determine the expression of specific markers of tumor cells in EMPD, we performed immunohistochemical (IHC) examination of markers in 17 patients with noninvasive or those with dermal invasion of EMPD. Although tumor cells in the epidermis and the dermis were positive for cytokeratins, including CK7 and CAM5.2, MUC1, CEA, and EMA, we determined the expression of another cytokeratin antibody, CK19, which has been only reported to be positive for EMPD cells.[14] Histopathological examination clearly demonstrated that in addition to the other antigens, CK19 was also a very useful marker of EMPD cells in the present study. HER-2 positivity has been reported in mammary Paget's disease (PD) or EMPD.[15-18] Moreover, EMPD patients with dermal invasion are expected to show greater HER-2 positivity than those without invasion; however, we did not observe any statistical significance by χ^2 test in our study. Recently, HER-2 is considered as a useful antibody for targeted therapy.[19] Although chromogenic in situ hybridization (CISH) revealed *HER-2* gene amplification,[20] no data on *HER-2* amplification

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Table 1. Immunohistochemical Characterization of Both Intraepidermal and/or Dermal Tumor Cells in Noninvasive or Invasive Extramammary Paget's Disease

Age	Gender	Location	CK7	CK19	CK20	CAM5.2	CEA	EMA	MUC1	Cyclin D1	*HER-2/FISH
Intraepidermal tumor cells in patients without invasion											
79	F	Genital	+	+	−	+	+	−	+	−	−
79	F	Genital	+	+	−	+	+	−	+	c+	1+
63	F	Genital	+	+	−	+	+	−	+	c+	1+
76	F	Genital	+	+	−	+	(+)	+	+	−	−
70	F	Genital	+	+	−	+	(+)	−	+	n(+)	1+/1.2
66	F	Genital	+	+	−	+	(+)	(+)	+	−	1+/1.1
84	M	Scrotal	+	+	−	+	−	−	+	−	−
63	M	Genital	+	+	−	+	−	+	+	−	−
59	M	Scrotal	+	+	−	+	−	−	+	−	−
87	M	Genital	+	+	−	+	−	+	+	c+	2+/1.2
57	M	Genital	+	+	−	+	+	+	+	c+	1+/1.5
63	F	Genital	+	+	−	+	(+)	(+)	(+)	n(+)	1+
63	F	Genital	+	+	−	+	(+)	(+)	(+)	n(+)	1+/1.1
Epidermal tumor cells in patients with invasion											
85	F	Genital	+	+	(+)	+	−	+	+	c+	1+/1.2
63	M	Scrotal	+	+	−	+	+	(+)	+	n(+), c+	2+/1.1
66	F	Genital	+	+	(+)	+	+	(+)	+	−	1+/1.3
75	F	Genital	+	+	−	+	−	+	+	−	1+
Dermal tumors cells in patients with invasion											
85	F	Genital	+	+	−	+	−	+	+	c+	1+
63	M	Scrotal	+	+	−	+	+	+	+	n(+), c+	2+
66	F	Genital	+	+	(+)	+	+	(+)	+	−	1+
75	F	Genital	+	+	−	+	+	+	+	−	1+

Abbreviations: F, female; M, male; +, positive; (+), focal positive; −, negative; n(+), focal nuclear positivity; c+, cytoplasmic positivity; *HER-2/FISH: HER-2 immunohistochemistry/fluorescence in situ hybridization titer.

obtained by fluorescence in situ hybridization (FISH) have been reported thus far. Therefore, we also performed FISH study using specimens immunohistochemically positive for HER-2 obtained from EMPD patients without invasion and those with dermal invasion.

Materials and Methods

We compared 4 patients with dermally invasive EMPD and 13 patients with noninvasive EMPD. We examined 17 patients (7 men and 10 women,) with ages ranging from 57 to 87 years (Table 1). The site of EMPD was the genital region in the case of 14 patients and the scrotum in the case of 3 patients (Table 1). The tumor was fixed in a 10% phosphate-buffered formalin solution and then most of the tumor was dissected into several slices, each of which was embedded in paraffin in a usual manner.

The dewaxed 4-μm sections were stained with hematoxylin–eosin (H&E). For mucin staining, diastase-digested periodic acid-Schiff (DPAS) reaction was also performed in some patients. IHC examination of representative sections definitely containing tumor cells was performed for detailed investigation. IHC staining was performed as reported[21,22] by using the labeled streptavidin–biotin (LSAB) 2 kit/horseradish peroxidase (HRP; Dako, Kyoto, Japan) with diaminobenzidine as the substrate for HRP and using antibodies as listed in Table 2.

FISH study was performed according to the procedure described in the manual of PathVysion HER-2 DNA probe kit (Abbott Molecular Inc, Japan), which was used for the examination of *HER-2* gene amplification in breast cancer.

Briefly, in each denatured DNA sample of tumor cells on deparaffinized sections, HER-2 DNA probe was hybridized with CEP 17 DNA probe. Then, after nuclear staining with 4′,6-diamidino-2-phenyl indole, positive HER-2 DNA (orange) and CEP17 DNA (green), were counted and analyzed under fluorescent microscope using MetaCyte scanning image cytometer (MetaSystems, Altlussheim, Germany). When orange signals were more than twice that of the green ones, the results of FISH were considered to be positive for amplification of *HER-2* gene. If the orange signals were less than twice that of the green ones, the results were considered to be negative.

Table 2. Antibodies Used

Antibody	Dilution	Pretreatment	Manufacturer
CK7	1:50	P	Dako
CK19	1:30	AC	Scytek
CK20	1:25	AC	Dako
CAM5.2	1:20	P	Becton–Dickinson
EMA	1:50	MW	Dako
CEA	1:25	P	Dako
MUC1	1:100	AC	BD Biosciences Pharmingen
HER-2	1:200	MW	Dako
Cyclin D1	1:50	AC	Dako
ER	1:50	AC	Dako
PGR	1:800	AC	Dako

Abbreviations: EMA, epithelial membrane antigen; CEA, carcinoembryonic antigen; HER2, HER-2/neu; ER, estrogen receptor; PGR, progesterone receptor; P, pronase; AC, autoclaving; MW, microwave.

Statistical study on HER-2 expression with IHC was done using χ^2 test, between EMPD patients without (13 cases) or with (4 cases) dermal invasion as shown in Table 1.

All patients participating in the present study have provided written informed consent, and the identities of each patient have been strictly protected.

Results

CK7, CK19, CAM5.2, and MUC1 were strongly positive antibodies for both intraepidermal and dermal invasion of tumor cells in all patients of 13/13 (100%) noninvasive and 4/4 (100%) dermal-invasive cases as shown in Table 1. However, detailed examination revealed that CK7 and CK19 were uniformly positive for all of tumor cells, including cells with dermal invasion (Figure 1). CK7- and CK19-positive cells showed distinct tumor margins in the epidermis and clearly revealed dermal invasion (Figure 2). On the other hand, CAM5.2 and MUC1 stained tumor cells very well, but stable staining of individual tumor cells in each patient was not possible. In some CAM5.2- and MUC1-positive patients, negative or weakly positive tumor cells were intermingled with the positive ones. CK20 was mostly negative although focally weak positive findings were found in only 2 patients (Table 1).

Tumor cells were positively stained for EMA and CEA, but some patients were negative for these antigens. EMA was positive in 7/13 (50%) patients without invasion and 4/4 (100%) patients with dermal invasion; CEA was positive in 9/13 (66.6%) patients and 2/4 (50%) patients without invasion and with dermal invasion, respectively. In addition, the stainability of these antigens was not stable even in the case of patients positive for these antigens (Figure 3). Cyclin D1 was positive only in a limited number of patients; a small number of nuclei were positive in 3/13 patients without invasion and 1/4 patients with dermal invasion. Estrogen receptor (ER) and progesterone receptor (PGR) were negative in 6 patients examined, including 4 patients without invasion and 2 with dermal invasion.

HER-2 was positive in 8/13 (61.5%) patients without invasion and in all of 4 patients (100%) with dermal invasion. The tumor cell membranes were positive in linear fashion, including dermally invasive tumor cells (Figure 4). Although we did not observe any statistically significant differences between HER-2 positivity in patients with and without dermal invasion according to χ^2 test ($P > .05$), it is of interest that all patients with dermal invasion were positive for HER-2, which is consistent with the findings of IHC examination. FISH study showed negative results for HER-2 antibody in 8 patients, including those without and with dermal invasion, who were immunohistochemically positive for HER-2 (Table 2). The FISH titers were from 1.1 to 1.5 (Figure 5), and none of 8 patients showed a FISH titer of more than 2 points (Table 1).

Discussion

Our study showed that CK7 and CK19 were the excellent markers and CAM5.2 and MUC1 were the good markers of EMPD cells. Our results also indicate that CK7 and CK19 are extremely useful to clearly identify the margin of EMPD cells. Each tumor specimen obtained from all the 17 EMPD patients examined was distinctly positive for CK19 and CK7. However, although CAM5.2 and MUC1 were good markers,[3,5] they were not consistently expressed in individual cancer cells. Detailed observation revealed that in the large foci of cancer cells, positive and negative tumor cells were intermingled. Thus, CK19 and CK7 were more useful than CAM5.2 and MUC1. CK7 is a good marker of tumor cells in EMPD,[3] but CK19 has not been reported as the marker of EMPD, except for one study.[14] We have definitely confirmed that CK19 is an excellent marker of EMPD. Thus, CK19 and CK7 uniformly stained the cancer cells, and both these cytokeratins may be useful to determine the tumor margins of EMPD in clinical practice.

EMA and CEA also stain cancer cells, and they are reported to be good markers of EMPD,[1,2] but in our study, approximately 80% of the patients without invasion and those with invasion were positive for these antigens. The patients who were negative for these antigens have been described in Table 1.

Recently, cyclin D1 has also been reported to be a useful marker for the EMPD cells.[11,12] However, our results showed that only a few nuclei of cancer cells in 5 patients were positive for this antibody. Thus, on the basis of our

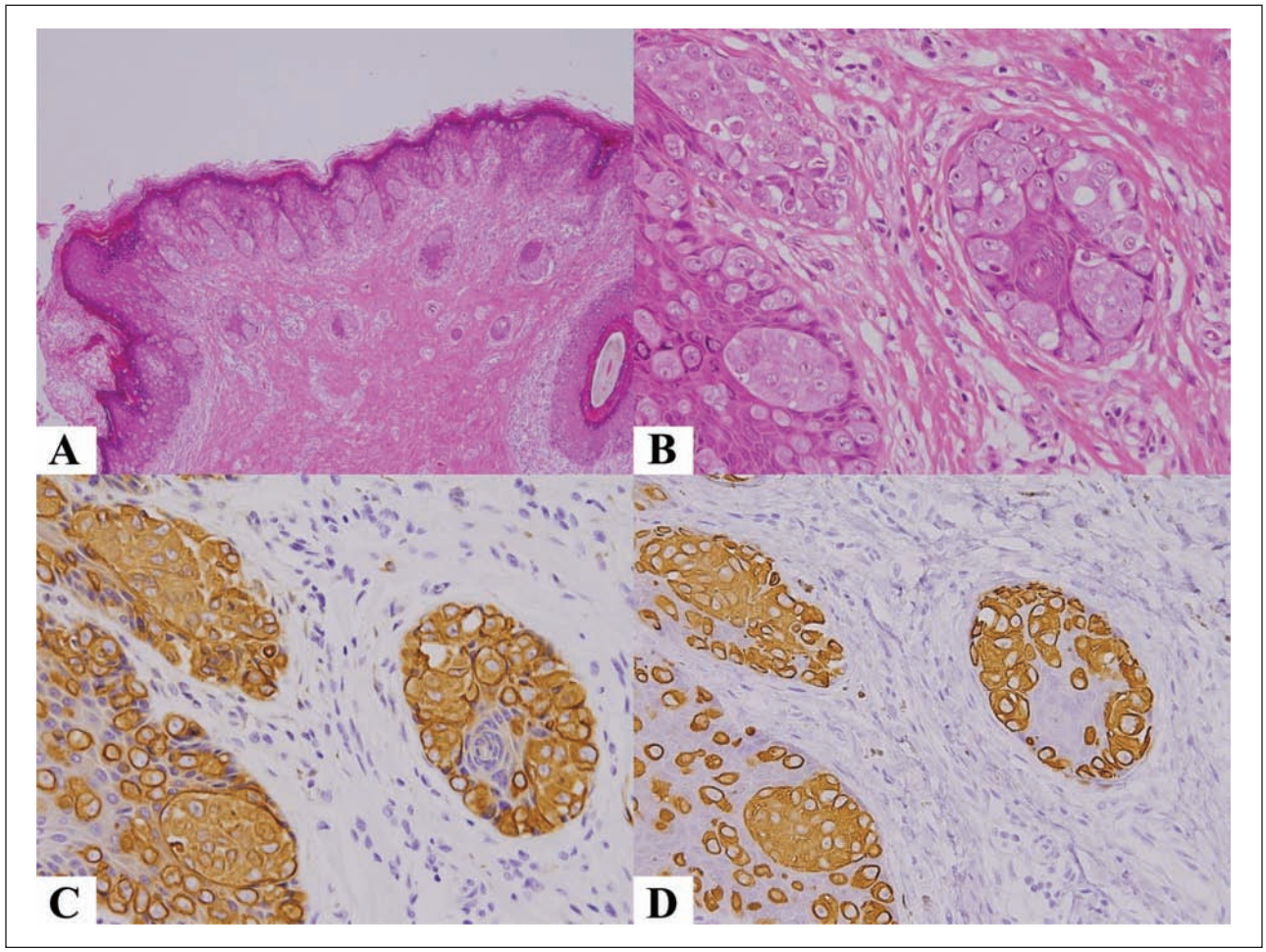

Figure 1. (A) Intraepidermal tumor cell proliferation, including hair follicle (magnification 10×). Serial sections stained by (B) hematoxylin–eosin (H&E; magnification 20×), (C) cytokeratin 7 (CK7; magnification 20×), and (D) CK19 (magnification 20×). Tumor cells were well stained in (C) and (D).

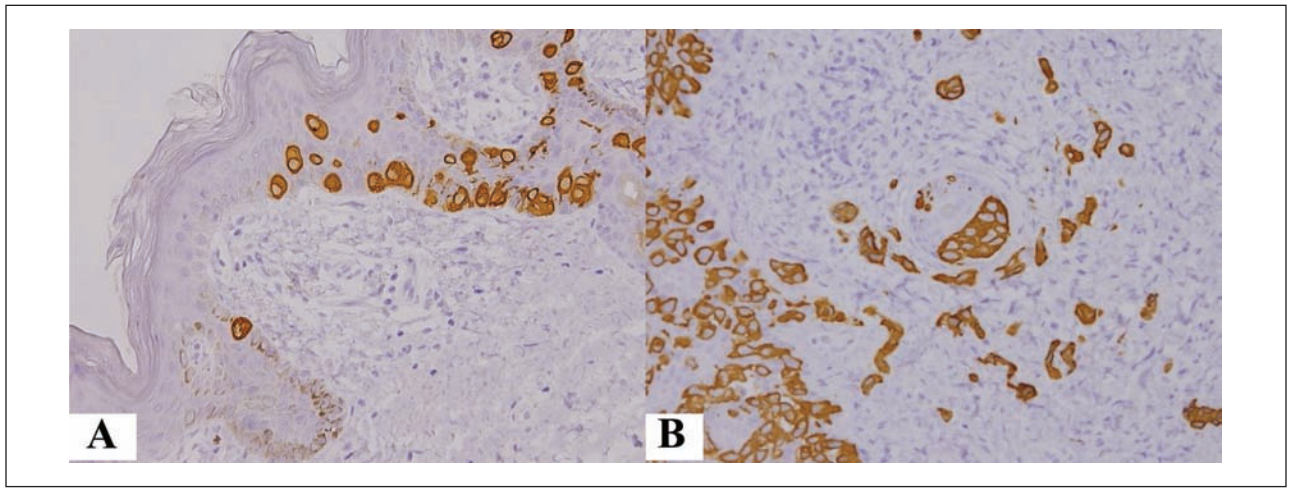

Figure 2. (A) Cytokeratin 19 (CK19) sharply stained the margin of intraepidermal tumor cell growth and (B) dermal invasion was clearly shown with CK19 stain. Magnification: A and B 200×

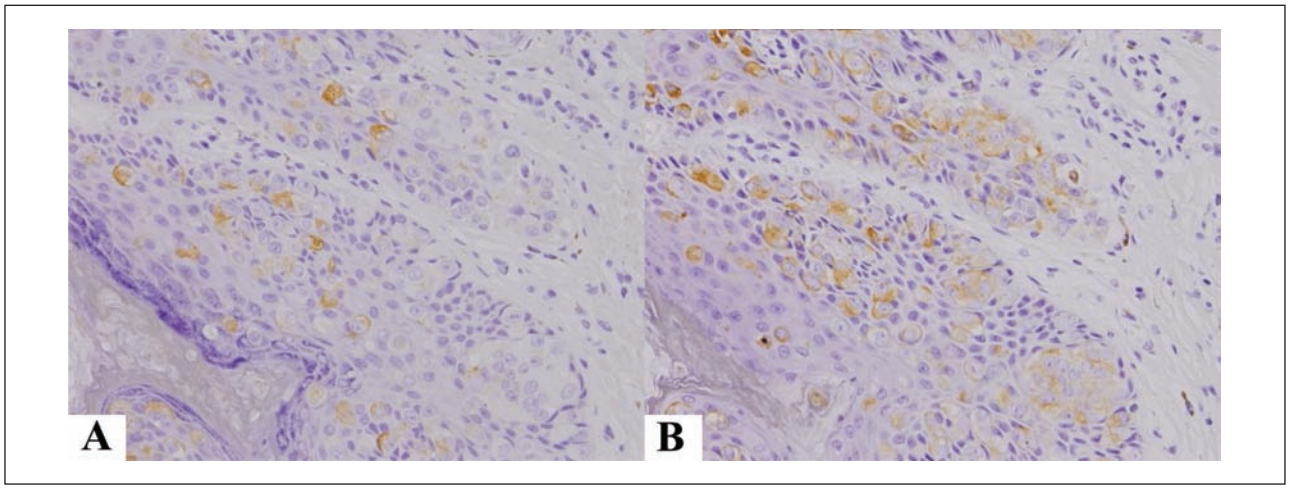

Figure 3. Uneven staining of intraepidermal tumors cells with (A) epithelial membrane antigen (EMA) and (B) carcinoembryonic antigen (CEA) stains. Note intermingled negative and/or positive cells (A and B; magnification 200×)

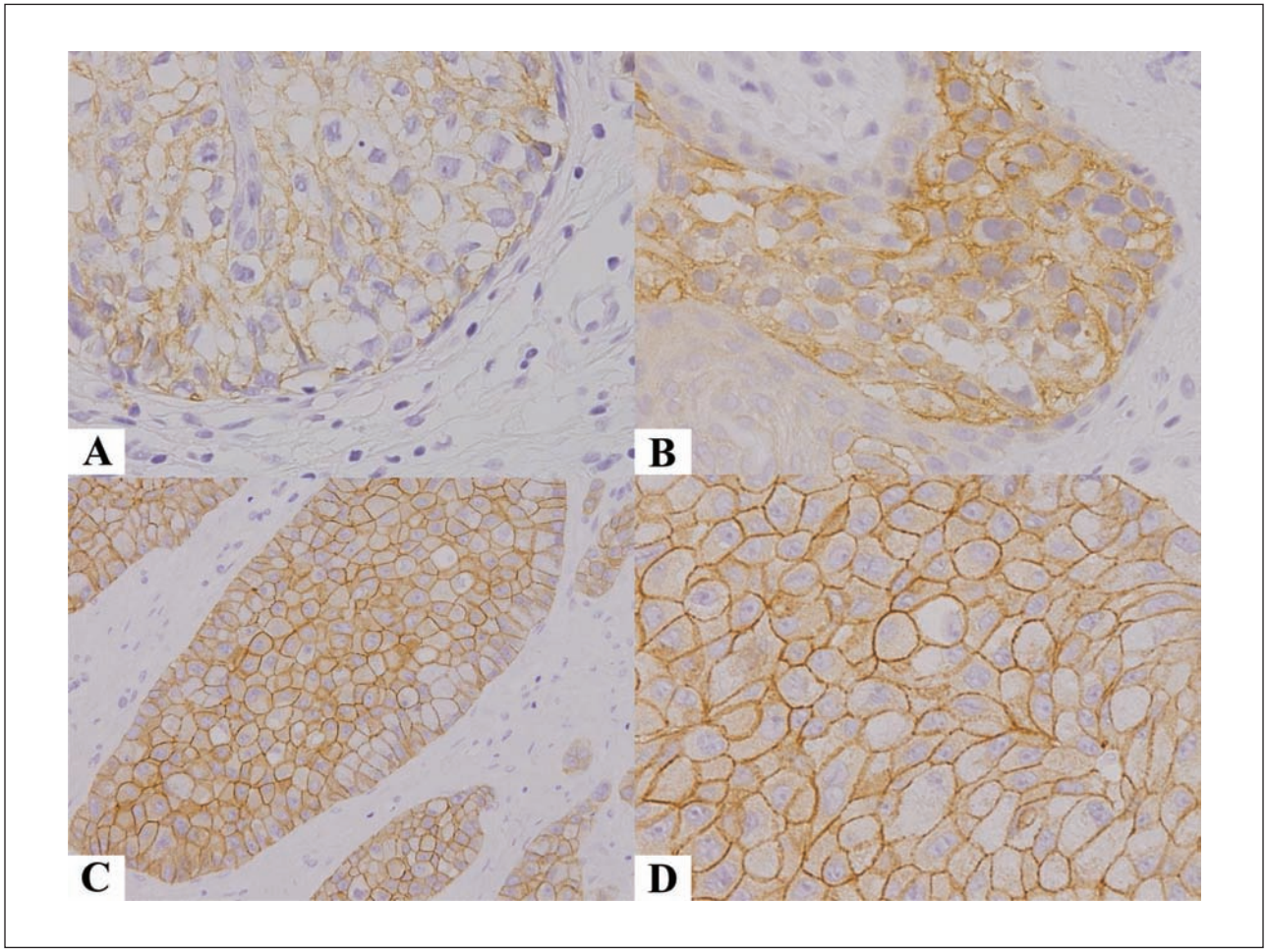

Figure 4. (A) 1+-positive and (B) 2+-positive intraepidermal tumor cells with HER-2 are shown in contrast with 2+dermally invasive tumor cells (C, D). Note positive immune reaction of tumor cell membranes. A-D: HER-2 stain; A, B, and D magnification 400× and C magnification 100×

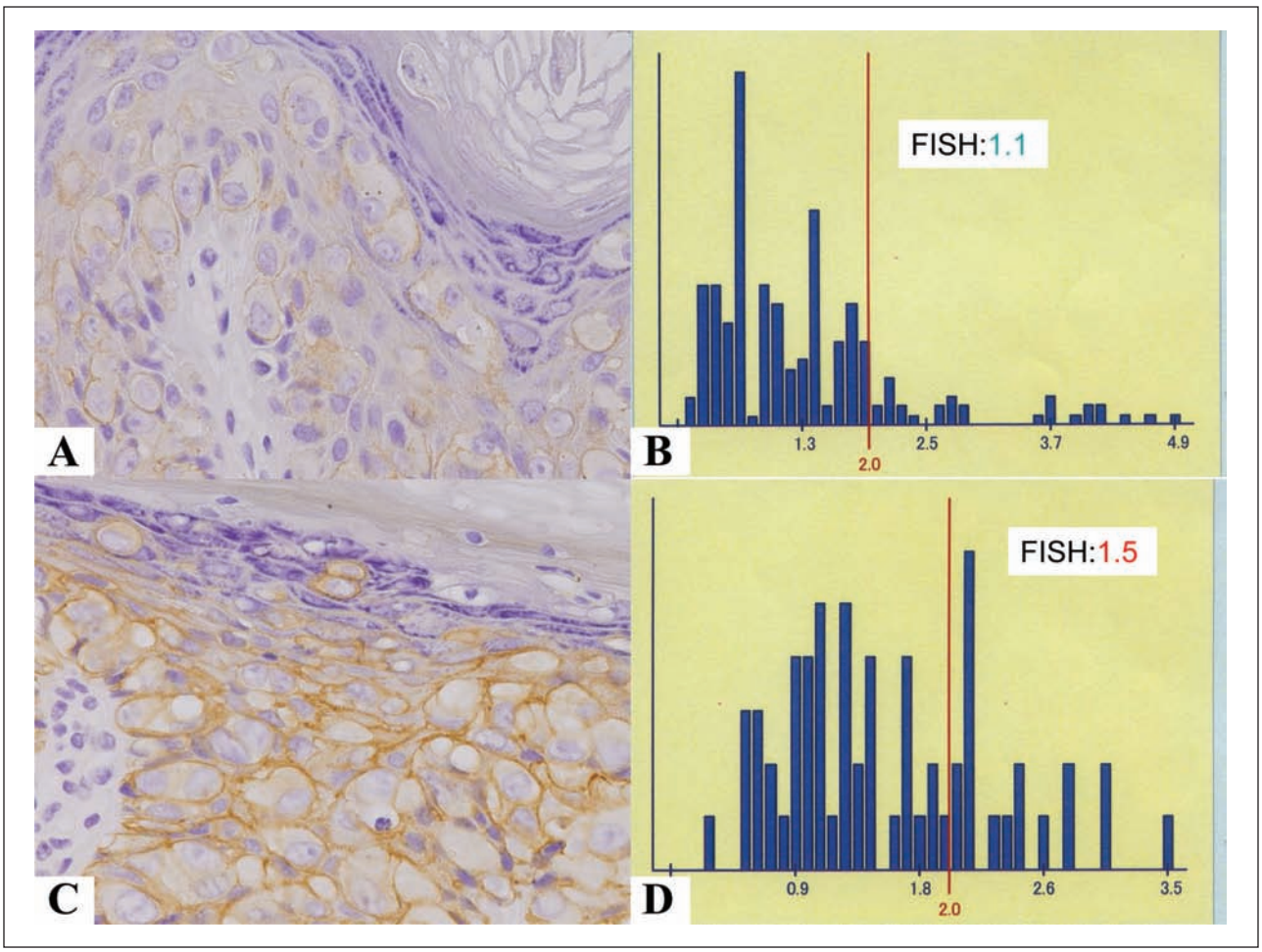

Figure 5. (A) 1+- and (C)2+- HER-2 positive patients were both negative with fluorescence in situ hybridization (FISH) study, as shown in B and D. Each was 1.1 and 1.5, respectively in titer. A and C: HER-2 stain, magnification 400×

results, cyclin D1 does not appear to be an excellent marker. Moreover, the cytoplasm of some cancer cells was stained with this antibody (Table 1).

Comparison of the origin of EMPD with that of PD indicated that intraepidermal ductal cells of the sweat glands were the site of origin of PD because of the positivity of GCDFP-15[8] and MFGM gp70[13] but not of MFGM gp155,[13] which was positive for intraductal cells of the mammary glands. Therefore, gp155 was positive in tumor cells of PD.[13] ER and PGR were negative for EMPD,[3] but positive for PD.[3] These results indicated that PD cells were derived from ductal cells of the mammary gland. Consistent with the above findings, ER and PGR were negative in 6 EMPD patients, including those with dermal invasion. Thus, the origins of PD and EMPD appeared to be different.

In the present study, we could not obtain the statistical differences on HER-2 positivity with IHC between noninvasive and invasive cases of EMPD. To obtain more accurate statistical difference, we need to accumulate more samples, especially of dermal invasive cases of EMPD.

Immune therapy using HER-2 antibody was considered to be useful because of its success in breast cancer.[23,24] Some studies have reported[15-20] strong expression of HER-2 by immunohistochemical examination.[15-19] More than 40% Japanese EMPD patients, including those with aggressive invasive or metastatic tumors, were positive for HER-2.[18] Although some patients are negative for intraepidermal cancer cells,[16] our study showed that 8 out of 13 patients without invasion and all patients with dermal invasion were positive for intraepidermal cancer cells, including IHC2+ according to the standard of breast cancer.

Although CISH showed positive results of *HER-2* gene amplification,[20] FISH study on EMPD has not been

performed. In the present study, FISH showed that all the 8 patients, including those without invasion or those with dermal invasion were negative for *HER-2* although they were definitely immunohistochemically positive for HER-2 on the membranes of cancer cells. Their positivity was IHC1+ to IHC2+ according to the standard of breast cancer. The interrelationship between IHC examination and FISH for breast cancer showed that IHC3+ patients were mostly positive with FISH; however, there were several exceptions in some patients. IHC 2+ cases contained FISH-negative cases between 25% and 48% according to the reports of breast cancer.[23-25] Thus, it remains to be clarified whether *HER-2* was truly amplified in our IHC1+ to IHC2+ patients with EMPD, which were negative for HER-2 by FISH. If *HER-2* is amplified, it might be useful for targeted therapy of EMPD as suggested in recent studies.[19]

Our results indicate that in addition to CK7, CK19 is an excellent marker of tumor cells in EMPD, and all patients with dermal invasion were positive for HER-2, although no *HER-2* gene amplification was obtained in FISH study in these patients.

Further studies are required on additional number of patients, especially for *HER-2* gene amplification, to determine whether targeted therapy using HER-2 can be performed for EMPD in the near future.

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Declaration of Conflicting Interests

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