Expression of Claudin-1, p53 and E-cadherin in Pseudoepitheliomatous Hyperplasia and Squamous Cell Carcinoma of the Head and Neck

Keum Ha Choi · Jae Hong Lim
Ju Hyung Lee[1] · Keun Sang Kwon[1]
Ho Lee[2] · Ho Sung Park
Myoung Ja Chung · Woo Sung Moon
Jae Soon Eun[3] · Dong Geun Lee
Kyu Yun Jang

Departments of Pathology, [1]Preventive Medicine and [2]Forensic Medicine, Chonbuk National University Medical School, Institute for Medical Sciences and the Center for Healthcare Technology Development, Jeonju; [3]College of Pharmacy, Woosuk University, Wanju, Korea

Corresponding Author
Kyu Yun Jang, M.D.
Department of Pathology, Chonbuk National University Medical School, San 2-20 Geumam-dong, Deokjin-gu, Jeonju 561-180, Korea
Tel: 063-270-3071
Fax: 063-270-3135
E-mail: kyjang@chonbuk.ac.kr

Background : Pseudoepitheliomatous hyperplasia (PEH) is a reactive proliferation of surface epithelium and can be confused with invasive squamous cell carcinoma (SCC) in head and neck biopsy specimens. To distinguish PEH from invasive SCC, immunohistochemical staining for claudin-1, E-cadherin and p53 was performed. **Methods :** Eighteen cases of PEH and 29 invasive SCC from head and neck lesions were immunostained and examined. **Results :** The invasive SCC showed increased staining of claudin-1 ($p<0.001$) and p53 ($p<0.001$) and decreased staining of E-cadherin ($p=0.005$) compared to the PEH specimens. The combined score calculated by adding the positive sum of claudin-1 and p53 and subtracting E-cadherin was useful for the differentiation of SCC from PEH (89.7% sensitivity and 88.9% specificity, $p<0.001$). **Conclusions :** The combined immunostaining for claudin-1, p53 and E-cadherin may help differentiate PEH from invasive SCC. The results of this study suggest that the increased expression of claudin-1 and p53 and the decreased expression of E-cadherin maybe markers for the aggressive growth of invasive SCC.

Key Words : Hyperplasia; Carcinoma, squamous cell; Claudin-1; p53 protein; E-cadherin

Pseudoepitheliomatous hyperplasia (PEH) is a reactive epithelial proliferation that occurs in response to underlying infectious, inflammatory or neoplastic conditions.[1] Histologically, PEH is characterized by epithelial hyperplasia with irregular cords of epithelial cells extending into the dermis, as well as varying degrees of hyperkeratosis and papillomatosis. Unlike squamous cell carcinoma (SCC), PEH lacks pronounced nuclear atypia, abundant or abnormal mitoses and prominent dyskeratosis.[2] However, the histological features of PEH may simulate well-differentiated, infiltrating SCC. Therefore, PEH can be confused with invasive SCC of head and neck biopsy specimens.[3,4]

The problem is in part due to small tissue samples, dense inflammation and poor orientation. Several immunohistochemical markers such as p53, E-cadherin, collagen IV, matrix metalloproteinase 1 (MMP-1), MMP-7, MMP-12, and MMP-13, have been studied for their association with the diagnosis of SCC. In addition, some markers have been found to be useful in the differentiation of SCC from PEH.[2,3,5-7] However, previous studies on the immunohistochemical staining patterns of SCC and PEH are limited.

E-cadherin is a cell membrane associated protein involved in cell-cell adhesion. The loss of expression of E-cadherin has been

reported in various malignant tumors, including SCC. It has also been suggested that the expression of E-cadherin and p53 are useful in differentiating SCC from PEH.[3] Claudins are a group of transmembrane proteins that are products of a gene family composed of more than 24 members;[8-10] increased expression of claudin-1 in SCC of the uterine cervix[11] and colon cancer[12] has been previously reported. To distinguish PEH from invasive SCC, we performed immunohistochemical staining for claudin-1, p53 and E-cadherin, followed by statistical analysis of the data.

MATERIALS AND METHODS

Patients and tissue samples

We retrospectively studied 18 cases of PEH and 29 of SCC that were obtained from 46 patients who had undergone mucosal biopsy, from the head and neck regions, between June 2002 and December 2005, and had follow up information for at least two years. This study had local ethics committee approval obtained from the Chonbuk National University Hospital's institutional review board. Informed consent was provided according to the Declaration of Helsinki.

We defined PEH as the characteristic proliferation downgrowth of rete pegs into the submucosa, where the individual cells mature with only occasional dyskeratosis.[2,3] In cases with SCC, surface mucosa and invasive tumor existed on the same slides. In cases with PEH, nonproliferative surface mucosa and hyperplastic mucosa existed on the same slides.

Immunohistochemical staining and scoring

The tissue sections were deparaffinized and treated using a microwave antigen retrieval procedure in sodium citrate buffer (pH 6.0) for 12 min. The tissue samples were immunostained with monoclonal antibodies against claudin-1 (1:100, Zymed laboratories, South San Francisco, CA, USA), p53 (1:50, Novocastra, Newcastle, UK) and E-cadherin (1:100, Zymed laboratories) with the avidin-biotin complex technique, using appropriate positive and negative controls.

The immunohistochemical analysis was performed by three authors with the results agreed on by consensus and without knowledge of clinical or pathological information. For the semi-quantification of claudin-1 and E-cadherin expression, a scoring system was developed by multiplying the intensity of the

staining by the area that was stained. The intensity of the cell staining for claudin-1 and E-cadherin was graded according to the following scale: 0, no staining; 1, mild staining; 2, moderate staining; 3, marked staining. The areas of staining for the claudin-1, E-cadherin and p53 were evaluated using the following scale: 0, <10% of the cells stained positive; 1, 10-29% of the cells stained positive; 2, 30-69% of the cells stained positive; 3, ≥70% of the cells stained positive. The maximum score for claudin-1 and E-cadherin expression was 9, and the minimum score was 0. Immunostaining for p53 was semi-quantified by the area of staining, which was scored from 0 to 3.

Statistical analysis

SPSS software (version 15.0) was used for statistical analysis. Comparison of the expression of claudin-1, p53 and E-cadherin for SCC and PEH were analyzed using the t-test and χ^2 test. The discriminatory power was assessed by the area under the curve (AUC) of the receiver operating characteristic (ROC) curve, and the best cutoff point was determined by the highest positive likelihood ratio (sensitivity/[1-specificity]) for each staining method. A p-value less than 0.05 was considered significant.

RESULTS

The average age of the patients with SCC was 65.2 ± 10.4 (range, 33-80 years), and for PEH it was 53.4 ± 18.8 (range, 8-84 years). The male-female ratio was 8:10 and 24:5 for PEH and SCC, respectively. The location of the sampled lesion was the lip and oral cavity (14 cases of SCC and 17 cases of PEH), pharynx (one case in both SCC and PEH), and larynx (14 cases of SCC) (Table 1).

We used ROC curves to determine the cutoff level and to compare the staining methods by the AUC. Since the ROC curve is a plot of the true positive rate (sensitivity) versus the false positive rate (1-specificity) for determination of the disorder (SCC in our study) or normal state (PEH in our study), the cutoff level for the ideal test is presented as the sensitivity 1 and specificity 1, or as the AUC 1.000. In this study, the cutoff levels for each stain were determined to be: six for claudin-1, one for p53 and four for E-cadherin. The AUCs were 0.900 for claudin-1, 0.896 for p53 and 0.744 for E-cadherin (Fig. 1) (Table 2). Depending on the cutoff level, each variable could be defined as positive (1) or negative (0). A higher score of claudin-1 and p53 combined, without E-cadherin, was more common in SCC

than PEH. A model was established with the sum of positive claudin-1 and p53 minus the number of positive E-cadherin (i.e., the combined score=claudin-1+p53-E-cadherin). The range of combined scores was -1 to 2, and the cutoff point was 1 (Fig. 1). Immunohistochemical staining for claudin-1, p53 and E-cadherin all showed significantly different staining patterns for SCC compared to PEH, and the most significant model was the one with the combined score. Because a higher AUC generally reflects a more effective diagnostic method, the combined score (AUC=0.945) was determined to be the most effective method to distinguish between SCC and PEH (89.7% of sensitivity and 88.9% of specificity, p<0.001) (Table 3).

Claudin-1 was detected in the cytoplasm membrane and showed increased membranous staining with invasive SCC compared to PEH (positive in 82.8% of SCC and 16.7% of PEH, p<0.001). For PEH, immunoreactivity to claudin-1 was limited to the cytoplasm membrane of the intermediate cells of the squamous epithelium, and the basal and parabasal layers were not immunoreactive (Fig. 2A). For SCC, the tumor cells showed strong immunoreactivity along the cytoplasmic membrane (Fig.

2B). Immunohistochemical reactivity for p53 was increased within the nuclei of the invasive SCC (positive in 82.8% of SCC and 11.1% of PEH, p<0.001). For PEH, nuclear staining for p53 was focally limited to the basal and parabasal cells (Fig. 2C). The SCC showed diffuse and strong immunoreactivity for p53 throughout the tumor (Fig. 2D). E-cadherin showed decreased
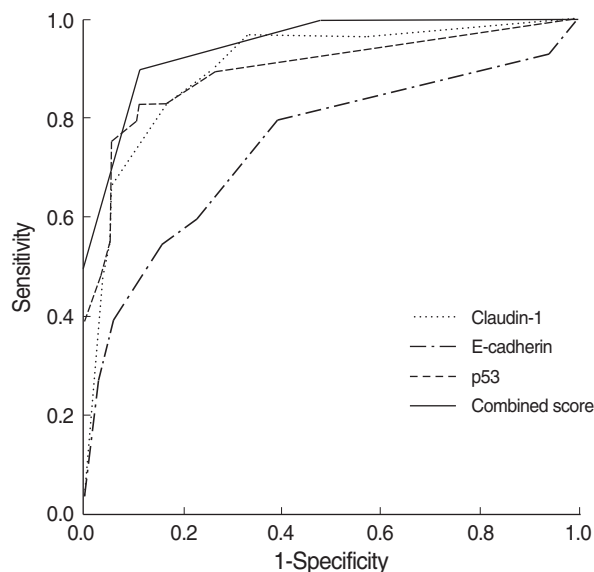

Fig. 1. Analysis of sensitivity and specificity for claudin-1 score, p53 score, E-cadherin score, and combined score between squamous cell carcinoma and pseudoepitheliomatous hyperplasia, represented by receiver operator characteristic curves.

Table 1. Distribution of cases by patient age, patient sex, and location of lesion

Characteristics	SCC (n=29)	PEH (n=18)
Age (range)	65.2±10.4 (33-80)	53.4±18.8 (8-84)
Sex		
Male	24 (82.8%)	8 (44.4%)
Female	5 (17.2%)	10 (55.6%)
Location		
Lip and oral cavity	14 (48.3%)	17 (94.4%)
Pharynx	1 (3.4%)	1 (5.6%)
Larynx	14 (48.3%)	0 (0.0%)

SCC, squamous cell carcinoma; PEH, pseudoepitheliomatous hyperplasia.

Table 2. Statistical data on receiver operating characteristic curves

Score	AUC	p-value	95% CI	Cutoff
Claudin-1*	0.900	<0.001	0.804-0.997	≥6
p53	0.896	<0.001	0.802-0.989	≥1
E-cadherin*,†	0.744	0.005	0.602-0.886	≤4
Combined score‡	0.945	<0.001	0.884-1.007	≥1

AUC, area under curve; 95% CI, 95% confidence interval.
*, the score was obtained by multiplying staining intensity score by staining area score; †, because the lower staining score of E-cadherin was presented in squamous cell carcinoma rather than pseudoepitheliomatous hyperplasia, the values were calculated reversely; ‡, combined score=claudin-1+p53-E-cadherin (variables were positive or negative by cut-off level).

Table 3. Expression of claudin-1, p53, and E-cadherin in squamous cell carcinoma and pseudoepitheliomatous hyperplasia

Characteristics	SCC (n=29) (%)	PEH (n=18) (%)	Sensitivity	Specificity	p-value*
Claudin-1					
Negative (≤4)	5 (17.2)	15 (83.3)	82.80%	83.30%	<0.001
Positive (≥6)	24 (82.8)	3 (16.7)			
p53					
Negative (0)	5 (17.2)	16 (88.9)	75.90%	94.40%	<0.001
Positive (≥1)	24 (82.8)	2 (11.1)			
E-cadherin†					
Negative (≤4)	23 (79.3)	7 (38.9)	79.30%	61.10%	0.005
Positive (≥6)	6 (20.7)	11 (61.1)			
Combined score‡					
Negative (≤0)	3 (10.3)	15 (83.3)	89.70%	88.90%	<0.001
Positive (≥1)	26 (89.7)	3 (16.7)			

SCC, squamous cell carcinoma; PEH, pseudoepitheliomatous hyperplasia.
*, analyzed by χ^2 test; †, because the lower staining score of E-cadherin was presented in SCC rather than PEH, the values were calculated reversely; ‡, combined score=claudin-1+p53-E-cadherin (variables were positive or negative by cut-off level).
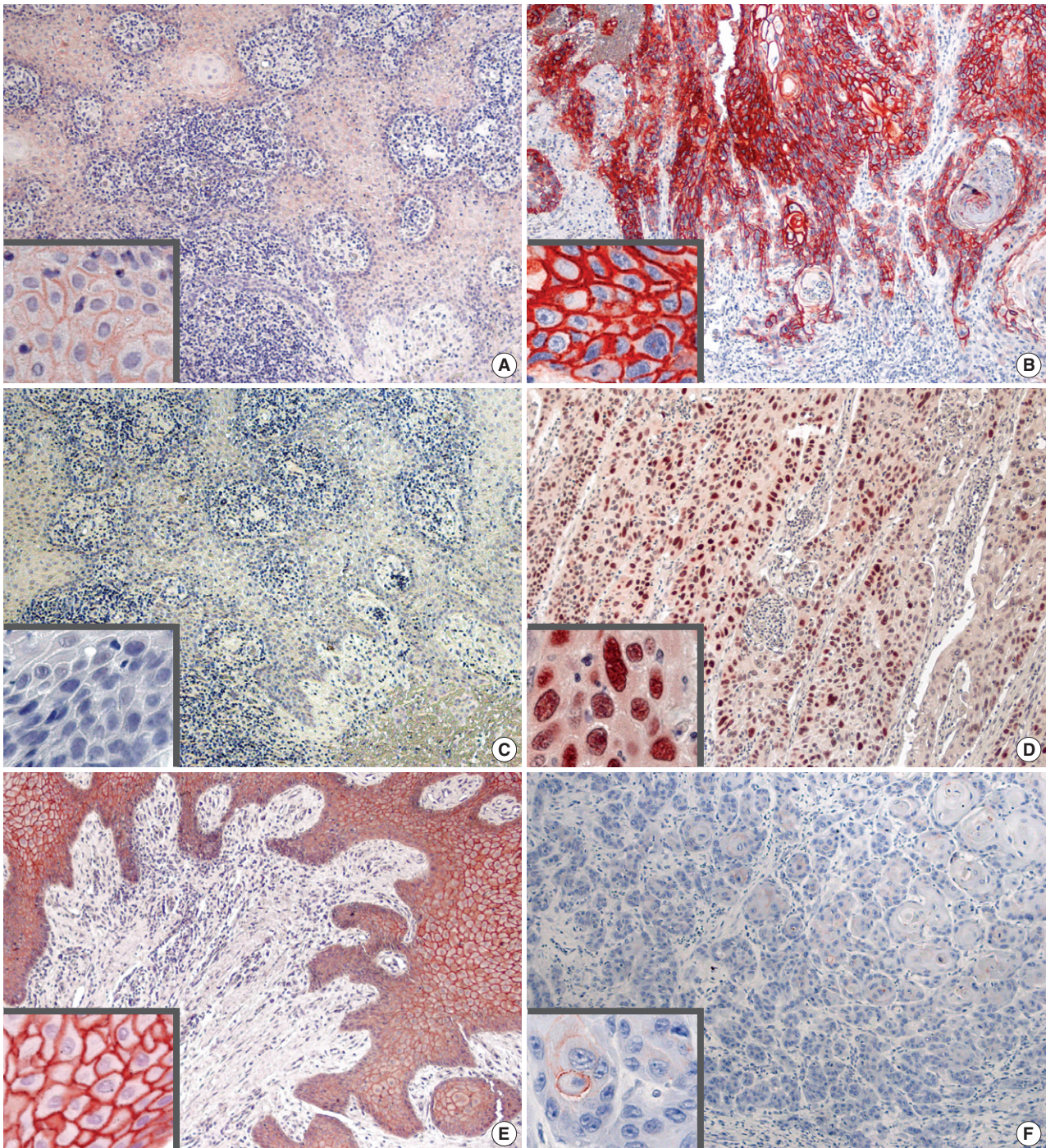
Fig. 2. (A) Pseudoepitheliomatous hyperplasia (PEH) shows weak staining for claudin-1 in intermediate cells. (B) Diffuse expression of claudin-1 along the cytoplasmic membrane of the squamous cell carcinoma (SCC). (C) Absence of immunoreactivity for p53 in PEH. (D) SCC shows numerous strongly positive nuclei for p53. (E) PEH shows uniform membranous staining for E-cadherin, even in irregular cords of epithelial cells extending into the dermis. (F) SCC shows weakly incomplete membranous staining for E-cadherin (A-F; ×100, inset; ×400).

membranous staining in the invasive SCC (negative in 79.3% of SCC and 38.9% of PEH, p=0.005). The benign epithelium of the PEH showed uniform membranous staining, even in the irregular cords of the epithelial cells extending into the dermis (Fig. 2E). The invasive tumor cells showed weak and incomplete membranous staining for E-cadherin (Fig. 2F).

DISCUSSION

Histologically, SCC can be distinguished from PEH by the presence of cytological atypia, including nuclear pleomorphism, maturation atypia and mitoses.[3,13] PEH does not demonstrate cytological signs of malignancy, although it may show reactive atypia.[3,13] In addition, PEH is recognized by its pattern of epidermal reaction to a variety of stimuli. Therefore, awareness of associated diseases and a careful search for an underlying condition is crucial for a reliable diagnosis.[13,14] However, it is sometimes extremely difficult to distinguish PEH from SCC and mistaking PEH for SCC may lead to the unnecessary removal of more tissue or unnecessary additional therapy, such as radiation therapy.[13,15,16] For this reason, some studies have tried to distinguish PEH from SCC by immunohistochemical staining.[3,7,17]

Immunohistochemical staining for E-cadherin, p53, MMP-1, MMP-7, MMP-12, MMP-13, MMP-19, and p16 have been suggested as useful in the differential diagnosis.[3,6,7] SCC has been reported to show significantly increased expression of p53, MMP-1, MMP-7, MMP-12, and MMP-13. Expression of E-cadherin, p16 and MMP-19 has been reported to be decreased in SCC.[3,6] On the other hand, immunostaining for collagen IV, MMP-3, MMP-8, MMP-9, MMP-10, or the number of CD1a-positive cells has not been useful for differentiating these two entities.[3,6,17] Therefore, we sought to differentiate PEH from the well-differentiated invasive SCC by immunostaining with claudin-1, p53 and E-cadherin. The results of this study showed that the sole expression of these markers, a combination of increased expression of p53 and claudin-1, and the decreased expression of E-cadherin, was useful in the differentiation of these two diseases. This is the first report to present a combined scoring system using several immunohistochemical markers to differentiated SCC from PEH.

The claudins are integral transmembrane proteins located at tight junctions.[8] The claudin family consists of at least 24 members, whose expression depends on the cell type and tissue.[8,18,19] The claudins participate in intracellular signaling[20] and may have a role in cancer development.[11,19] Increased expression of claudin-1 has been demonstrated in intraepithelial neoplasia and SCC of the uterine cervix compared to normal epithelium; these findings have suggested that claudin-1 might be a good diagnostic marker for the detection of cervical intraepithelial neoplasia.[11] Moreover, pulmonary SCCs showed a significantly higher expression of claudin-1 compared to pulmonary adenocarcinoma; therefore, this expression pattern might provide an additional diagnostic tool.[21] The results of our study also showed that claudin-1 expression was significantly increased in SCC compared to PEH. Taken together, these data suggest that increased expression of claudin-1 plays an important role in the development of SCC. In addition, increased expression of claudin-1 has been reported in colorectal adenocarcinoma.[12] However, there are some differences in the expression among different tumor types; invasive breast carcinoma and malignant melanoma, for example, showed decreased expression of claudin-1.[22,23]

E-cadherin is a cell-cell adhesion transmembrane molecule that plays an important role in both cell adhesion and cell signal transduction. Various human malignant tumors, such as carcinomas of the breast, esophagus, colon, and stomach, have been reported to have decreased expression of E-cadherin. Moreover, decreased expression of E-cadherin has been associated with vascular invasion and decreased survival in head and neck SCCs.[24] Furthermore, consistent with our findings, decreased expression of E-cadherin has also been helpful in distinguishing SCC from PEH.[3]

p53 is one of the most common tumor suppressor genes found in human malignancies; approximately 50% of human malignancies show alteration of this gene.[25] In paraffin-embedded tissue, malignant tumors show more frequent expression of p53 when compared to their benign counterparts.[2,3,26] However, despite the frequent alteration of the *p53* gene in malignant tumors, immunohistochemical expression rates of p53 vary in different studies. Our study showed that 82.8% of SCC and 11.1% of PEH tissues were positive for p53. Our results are consistent with other reports.[3,26] It has been reported that 81-87% of SCC tissues express p53, and that the p53 expression pattern is helpful in differentiating SCC from PEH.[3,26] Only 29% of PEH tissues were reported to have p53 expression,[3] with a less intense and more extensive staining pattern compared to SCC.[2] However, some reports have demonstrated that only 34-52% of SCC were immunoreactive for p53.[27,28]

In this study, the sole expression of claudin-1, p53 or E-cadherin also showed significantly different expression patterns between SCC and PEH. Furthermore, a combined score was most accurate in differentiating the two diseases, with 89.7% of sensitivity and 88.9% of specificity. However, despite the high sensitivity and specificity of the combined score, there were also false positive and false negative cases. Three of 29 (10.3%) SCC samples had a negative combined score and three of 18 (16.7%) PEH had a positive combined score. However, for difficult diagnostic cases, four out of 18 that were finally confirmed to be PEH had follow-up recommended or another biopsy due to the difficulty in differentiating PEH from SCC; two cases required anoth-

er biopsy. In our study, the combined score of these four cases was zero, zero, zero, and one. In other words, three of the four difficult to diagnose PEH cases had a negative combined score and one case had a positive combined score. This result suggests that despite the presence of false positive or false negative cases, the application of our immunohistochemical staining panel was very useful for differentiating PEH from SCC.

In conclusion, the combined immunostaining for claudin-1, p53, and E-cadherin was useful for differentiating pseudoep-itheliomatous hyperplasia from invasive squamous cell carcinoma. The results of this study suggest that increased expression of claudin-1 and p53 and decreased expression of E-cadherin may be markers for the aggressive growth of invasive squamous cell carcinoma. However, the combination of histological findings and immunohistochemical staining results is required for reliable differentiation of SCC and PEH. An adequate sample that can be properly oriented with hematoxylin-eosin-staining of the histological specimen remains the gold standard for a diagnosis.

REFERENCES

1. Sagi A, Zirkin H, Mahler D. Atypical pseudoepitheliomatous hyperplasia in squamous cell carcinoma in chronic wounds. Plast Reconstr Surg 1984; 74: 315-6.

2. Lee YS, Teh M. p53 expression in pseudoepitheliomatous hyperplasia, keratoacanthoma, and squamous cell carcinoma of skin. Cancer 1994; 73: 2317-23.

3. Zarovnaya E, Black C. Distinguishing pseudoepitheliomatous hyperplasia from squamous cell carcinoma in mucosal biopsy specimens from the head and neck. Arch Pathol Lab Med 2005; 129: 1032-6.

4. Oh HK, Kim JK, Park KK. Nasal type NK/T cell lymphoma with pseudoepitheliomatous hyperplasia: a case report. Korean J Pathol 2006; 40: 250-4.

5. Al Kassam D, Alvarez Marcos C, Blanco I, de Los Toyos JR, Llorente JL. Diagnostic value of E-cadherin, MMP-9, activated MMP-13 and anti-p53 antibodies in squamous cell carcinomas of head and neck. Med Clin (Barc) 2007; 129: 761-5.

6. Impola U, Jeskanen L, Ravanti L, *et al.* Expression of matrix metalloproteinase (MMP)-7 and MMP-13 and loss of MMP-19 and p16 are associated with malignant progression in chronic wounds. Br J Dermatol 2005; 152: 720-6.

7. Jeong Y, Sung KH, Lee SK. Immunohistochemical expression of MMP-7 and MMP-13 in chronic cutaneous ulcers and squamous cell carcinomas. Korean J Dermatol 2006; 44: 1284-9.

8. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. Prog Biophys Mol Biol 2003; 81: 1-44.

9. Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci U S A 1999; 96: 511-6.

10. Tebbe B, Mankertz J, Schwarz C, *et al.* Tight junction proteins: a novel class of integral membrane proteins. Expression in human epidermis and in HaCaT keratinocytes. Arch Dermatol Res 2002; 294: 14-8.

11. Sobel G, Paska C, Szabo I, Kiss A, Kadar A, Schaff Z. Increased expression of claudins in cervical squamous intraepithelial neoplasia and invasive carcinoma. Hum Pathol 2005; 36: 162-9.

12. Kinugasa T, Huo Q, Higashi D, *et al.* Selective up-regulation of claudin-1 and claudin-2 in colorectal cancer. Anticancer Res 2007; 27: 3729-34.

13. Scott G, Chen KT, Rosai J. Pseudoepitheliomatous hyperplasia in Spitz nevi. A possible source of confusion with squamous cell carcinoma. Arch Pathol Lab Med 1989; 113: 61-3.

14. Biswas A, van Pittius DG, Stephens M, Smith AG. Recurrent primary cutaneous lymphoma with florid pseudoepitheliomatous hyperplasia masquerading as squamous cell carcinoma. Histopathology 2008; 52: 755-8.

15. Calhoun KH, Wagner RF Jr, Kumar D, Hokanson JA. Pseudoepitheliomatous hyperplasia mistaken for cancer after delayed reconstruction. South Med J 1995; 88: 454-7.

16. Gacek MR, Gacek RR, Gantz B, McKenna M, Goodman M. Pseudoepitheliomatous hyperplasia versus squamous cell carcinoma of the external auditory canal. Laryngoscope 1998; 108: 620-3.

17. Galan A, Ko CJ. Langerhans cells in squamous cell carcinoma vs. pseudoepitheliomatous hyperplasia of the skin. J Cutan Pathol 2007; 34: 950-2.

18. Katoh M, Katoh M. CLDN23 gene, frequently down-regulated in intestinal-type gastric cancer, is a novel member of CLAUDIN gene family. Int J Mol Med 2003; 11: 683-9.

19. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2001; 2: 285-93.

20. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell 2004; 15: 176-88.

21. Paschoud S, Bongiovanni M, Pache JC, Citi S. Claudin-1 and claudin-5 expression patterns differentiate lung squamous cell carcinomas from adenocarcinomas. Mod Pathol 2007; 20: 947-54.

22. Cohn ML, Goncharuk VN, Diwan AH, Zhang PS, Shen SS, Prieto VG. Loss of claudin-1 expression in tumor-associated vessels correlates with acquisition of metastatic phenotype in melanocytic neoplasms. J Cutan Pathol 2005; 32: 533-6.

23. Tokes AM, Kulka J, Paku S, *et al*. Claudin-1, -3 and -4 proteins and mRNA expression in benign and malignant breast lesions: a research study. Breast Cancer Res 2005; 7: R296-305.

24. Kurtz KA, Hoffman HT, Zimmerman MB, Robinson RA. Decreased E-cadherin but not beta-catenin expression is associated with vascular invasion and decreased survival in head and neck squamous carcinomas. Otolaryngol Head Neck Surg 2006; 134: 142-6.

25. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994; 54: 4855-78.

26. Batinac T, Zamolo G, Jonjic N, Gruber F, Petrovecki M. p53 protein expression and cell proliferation in non-neoplastic and neoplastic proliferative skin diseases. Tumori 2004; 90: 120-7.

27. Oliveira LR, Ribeiro-Silva A, Zucoloto S. Prognostic significance of p53 and p63 immunolocalisation in primary and matched lymph node metastasis in oral squamous cell carcinoma. Acta Histochem 2007; 109: 388-96.

28. Shin DM, Lee JS, Lippman SM, *et al*. p53 expressions: predicting recurrence and second primary tumors in head and neck squamous cell carcinoma. J Natl Cancer Inst 1996; 88: 519-29.