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Detection of basement membrane alterations in head and neck tissue biopsy using double immunostaining technique

Churaiorn Unhasuta, Koson Intarasut

ABSTRACT

Certain cases pose diagnostic difficulty when it comes to discriminating between early squamous cell carcinoma, reactive processes, and dysplasia located at the head and neck. The gold standard test is confirming invasion of tissues beyond basement membrane. This study sought to verify whether basement membrane markers and usage of cytokeratin double immunostaining can improve the process. In total, forty retrospective cases of both neck and head biopsies have been used as the basis for this study. Out of the total, there were fifteen invasive squamous cell carcinoma cases (SCC), fifteen in-situ carcinoma cases (CIS), and ten pseudoepitheliomatous hyperplasia cases (PEH). The entire case distribution has received evaluation via immunohistochemistry. For this purpose, Cytokeratin (CK), Laminin and Collagen IV monoclonal antibodies have been used. In all cases, it has been found that the surroundings of the invasive tumor, and especially those of the malignant cells at the front, showed discontinuities or total absence of laminin and collagen IV staining. Therefore, it has been concluded that laminin and collagen IV may be used in the future as secondary differential diagnostic criteria besides double immunostaining for histological differentiation between SCC, CIS, and PEH.

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INTRODUCTION

Squamous cell carcinoma occurring at the neck and head regions is among the worldwide leading causes of mortality among cancer patients. Some of the major risk factors for SCC are heavy smoking, the use of tobacco products, betel quid oral consumption and alcohol consumption. In 2010, more than 26,000 citizens in Thailand were found with neck and head carcinomas. Despite the plethora of medical diagnosis and treatment advancements, morbidity and mortality rates for these types of cancer remain high [1]. This type of tumor typically commences its development in the moist squamous tissues lining the inner mucosal surface of the neck and head [2]. Although it is highly influenced by variability between various observers, the histologic biopsy examination of specific lesions is still used as the gold standard test.

One of the most used current techniques, namely that of light microscopy, is still difficult to practice at high accuracy rates because of the low threshold of detection of intraepithelial neoplasia microscopic invasion foci. This difficulty is often times amplified by the infiltration of these tissues by dense inflammatory material obscuring

the lesion. In addition, the cauterization and crushing used to dislodge biopsies from the lesions lead to very small specimens.

Basement membrane (basal lamina) is a specialised form of extracellular matrix consists of a mixture of collagens, laminin (glycoprotein), perlecan (heparansulphate glycoprotein) andentactin (glycoprotein). Basement membranes appear to be crucial in tumor invasion and metastasis. Loss of basement membrane has been detected in many types of carcinomas. The staining of laminin and discontinuous staining of Type IV collagen have been demonstrated for transformed epithelial invasion[1].

In the routine work up with Hematoxylin and Eosin stain, when pathologists are faced with lesions that they cannot make a decision between benign and malignancy ("Gray zone cases"). They usually sign out with a versus condition because they do not have the confidence to guide surgeons as to which way to go and that may be making the surgeons more confused.

There is a previous research about basement membrane in the double immunostaining technique but, they studied it in cervical cancer [3]. As immunohistochemical techniques have advanced, pathologists showed a propensity toward using staining for basement membrane components as secondary to light microscopy when dealing with invasion localized at the tissue surface [3]. However, the mere absence of basement membrane components at the site cannot rule out invasion much as the presence cannot confirm it. On the other hand, stromal invasion may be convincingly proven via double immunostaining for basement membrane components and cytokeratin, which aids visualization of the basement membrane breach by dysplastic epithelial cells.

This study is a qualitative retrospective analysis. The criteria of the cases we studied were based on previous diagnosis (with no doubts) of each category such as normal, PEH, CIS, and SCC to learn how these cases have alterations of basement membrane in steps from normal to become cancer. The objective of this paper is to study that the double immunostaining technique might be of value to add it in the routine of Head and Neck tissue biopsy to guide pathologists with more confidence to sign out the diagnosis and provide the surgeons with more information.

MATERIALS AND METHODS

Case selection

A 2006–2016 search of the surgical pathology patient files at the Institute of Pathology, Ministry of Public Health, Bangkok, Thailand was used to identify the target cases for this research.

Selection basis

1. All patients investigated for suspicion of head and neck squamous intraepithelial carcinoma were included. The search yielded 15 invasive squamous cell carcinoma patients, 15 in-situ carcinoma patients, and 10 pseudoepitheliomatous hyperplasia patients.
2. The second basis for inclusion criterion was that the case must have complete clinical history from a pre-biopsy period.

Exclusion basis

1. All cases where histopathological analysis showed non-squamous lesions.
2. Cases where patients have undergone prior radio or chemotherapy for lesions on the neck and head.

All samples were submerged in a 10 percent formalin solution and then embedded in paraffin at the Institute of Pathology. For each case, the most suitable paraffin blocks have been chosen for the immunohistochemical study. Diagnosis was established based on review of slides stained with hematoxylin and eosin (H&E).

Immunohistochemical analysis

A Leica Microsystems Bond maX machine, manufactured by Leica Microsystems, has been used during the immunohistochemical analysis of all the samples. The first step of the preparation was to incubate all samples at 60°C for an hour. The staining antibody application was made in the following order: firstly, collagen IV was applied in a 1:300 concentration, followed by the CIV22, Cell Marque, cat.no 239M-15[4], or Laminin antibody 1:100; afterward, the clone LAM-89, Novocastra, cat.no NCL-LAMININ followed [5].

The slides have been incubated at 37°C for ten minutes in Leica Bond enzyme 1 with the purpose of retrieving epitopes. The Leica Bond Polymer Refine Detection kit was used in immunohistochemical analysis. This was done in three steps and using immunoperoxidase. Samples were submerged for 40 minutes in primary antibody at room temperature, procedure which was followed by rise with Leica Bond Wash Solution done three times. Afterwards samples were submerged for five minutes into peroxide block 3 percent, and undergone three rinses with Leica Bond Wash Solution. An eight minute dip into Leica post primary polymer was then followed by other three rinses with the Bond Wash. The next eight minute dip was made with Leica poly-HRP IgG, which went through three Bond Wash rinses and through one rinse with deionized water. Afterward, a four minute dip into diaminobenzidine chromogen was rinsed three times with deionized water.

The second stain procedure employed usage of Cytokeratin antibody 1:600 and clone AE1/AE3, Novocastra, cat.no NCL-L-AE1/AE3. The samples were submerged into the antibody solution at room temperature for forty minutes. Afterward, a triple rinse with the Bond Wash followed. The Leica post primary polymer was used for dipping for twenty minutes and was triple rinsed with the Bond Wash. A twenty minute dip in Leica poly-AP IgG followed and was rinsed with the Bond Wash for three times, then with deionized water. Finally, the procedure was completed with a Leica mixed ted refine chromogen ten-minute application which was triple rinsed with deionized water. A five minute counterstain procedure followed via hematoxylin.

The criteria for staining intensity were as follows: no staining=negative; positive staining in light yellow color=weak staining; positive staining in yellow brown color=moderate staining; and positive staining in brown color=strong staining.

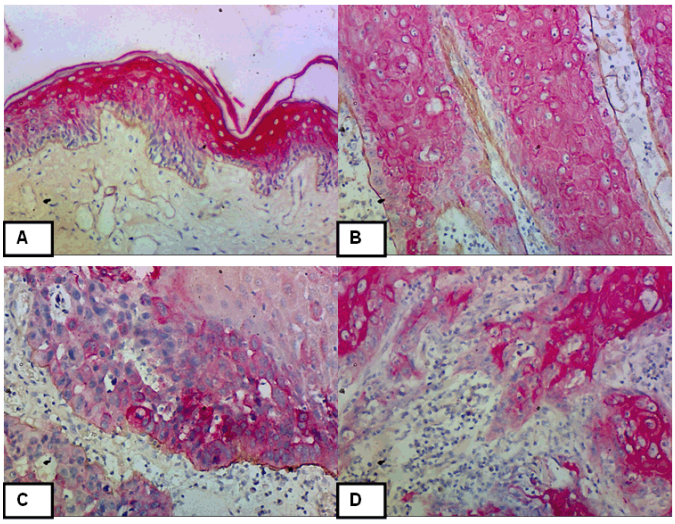

Figure 1(A–D): Laminin and CK double staining. (A) Normal cells and BM (magnification x100). (B) Chronic inflammation due to PEH, with intact BM (magnification x100). (C) BM showing lack of continuity and CIS (magnification x100). (D) No BM staining and SCC (magnification x100).

RESULTS

Epithelial tissue stained red in response to CK immunostaining with alkaline-phosphatase complex on a substrate composed of BT red reagent. The diaminobenzidine liquid chromogen immunostaining testing for collagen IV and laminin resulted in basement membrane turning into a brown stain (Figure 1A–1D). The staining of CK-laminin was not significantly different from CK- collagen IV. The laminin stain showed a membrane with a somewhat higher thickness.

In the cases of normal epithelial cells and PEH, the double immunostaining procedure revealed a continuous strong staining basement membrane (Figure 1A and 1B). In PEH, the membrane was always continuous, as revealed by the laminin and collagen IV staining intensity. All angle sections of the basement membrane showed lower stain intensity and an apparent thickening.

CIS staining revealed discontinuities in the basement membrane (Figure 1C) but also continuous portions. Invasive SCC caused immunoreactivity variability ranging from completely absent (negative) in areas with average to poorly differentiated tumors (Figure 1D) to discontinuous in well differentiated ones. In all cases, the invasive cell front of the malignant tumor were associated with absent or interrupted laminin and collagen IV staining. Rare cases were associated with clearly present and continuous basement membrane pushing against the malignant front. Yet a close examination of multiple biopsies found small irregular tumor nests and tiny single cells below the BM front, in the stroma. These areas were found through CK staining. The stroma of some cases manifested strong inflammatory infiltration, which hindered the evaluation of tumor size and hidden nests. The invasive malignant tissue in the infiltration was evidenced by means of CK staining, which led to a better measurement of tumor size.

DISCUSSION

PEH arises at cutaneous epithelium level as a proliferative reactive process. From a pathological standpoint, PEH is made possible by the secretion of tumoral cytokines. Ultimately, it triggers a hyper-proliferation of epithelial cells. Typically, inflammation is provoked by any type of injury but the organism removes affected tissue in a matter of hours. Due to immune responses, connective tissue proliferation is enabled by growth of vascular tissue and fibroblast. The last step of the process is epithelial complete regeneration and fibrous maturation [6]. Should this process be interrupted, the epithelium will exhibit a disorderly SCC-mimicking morphology which is in fact PEH.

Detection of tumoral invasion foci in intraepithelial neoplasms is incompletely supported by light microscopy, which is insufficient as a detection technique. In light microscopy, micro-invasion is difficult to visualize while BM continuity through singly antibody staining is also unreliable. The microinvasion detection through double immunostaining technique for CK and BM may aid detection because it highlights the regions where epithelial cells have crossed the boundary of the BM.

Via immunoperoxidase, an important component of the BM was highlighted in staining. Reactive and benign hyperplastic squamous mucosa is highlighted through continuous staining. In in-situ carcinoma and advanced dysplasia, the BM lacks continuity and shows signs of thinning [7]. Invasive tumors also show variable BM

distributions. Both laminin and collagen IV may react normally in tumors with clear borders and continuous stromal invasion. However, irregularly-corded invasive carcinomas and single malignant cells in the stroma have no BM at the domain wall of tumor and stroma [8]. The appearance of discontinuities in subepithelial BM occurs at the same time as the development of epithelial neoplastic transformation, as it is indicated by the changes in the laminin and collagen IV of pre- and malignant lesions [9]. Therefore, BM disappearance is not trivially an indicator of invasion.

Time savings and patient wellness considerations suggest using immunohistochemical analysis for finding invasion free regions. Similarly, detecting invasion in difficult cases leads to a swifter, better way to treatment. Even with severe inflammation obscuring the area, CK staining aided identification of invasive malignant cells [10]. Thus, CK staining improves infiltration detection accuracy, leading to a more accurate prognosis. The authors suggest evaluation via immunohistochemical methods mostly in cases suspected of minimal invasion. This study shows how BM characteristics and presentation varies with different types of malignant tissue and that these can produce BM.

Therefore, widely spread tumors may yet be associated with relatively intact BM around their infiltrate nests. These results suggest that an absent BM is not necessarily indicative of malignant invasion and its presence around tumors is not necessarily proof of a benign lesion [11]. Instead, the invasion aggressiveness is given by the BM crossing potential of the tumor cells. This process occurs in minimally invasive carcinoma and can be visualized well through the methods described here.

CONCLUSION

In doubtful cases, some helpful biomarkers have been identified as a secondary line of differential diagnosis. However, no one marker can be used as the sole criterion for establishing the nature of squamous intraepithelial tissue. Instead, a combination of markers must be used together with morphology, the second being critical in the way to diagnosis. Suitable treatment relies on sufficient excision and biopsy depth in malignant cases. A close collaboration between pathologists and clinicians is critical for delivering treatment.

REFERENCES

1. Singchat W, Hitakomate E, Rerkarmnuaychoke B, et al. Genomic alteration in head and neck squamous cell carcinoma (HNSCC) cell lines inferred from karyotyping, molecular cytogenetics, and array comparative genomic hybridization. PLoS One 2016 Aug 8;11(8):e0160901.
2. Head and neck cancer—patient version. [Available at: https://www.cancer.gov/types/head-and-neck]
3. Rush D, Hyjek E, Baergen RN, Ellenson LH, Pirog EC. Detection of microinvasion in vulvar and cervical intraepithelial neoplasia using double immunostaining for cytokeratin and basement membrane components. Arch Pathol Lab Med 2005 Jun;129(6):747–53.
4. MERCK. Collagen type IV (CIV22) mouse monoclonal antibody. [Available at: https://www.sigmaaldrich.com/catalog/product/sigma/239m1?lang=en&region=TH]
5. Leica. NovocastraTM lyophilized mouse monoclonal antibody laminin. [Available at: https://www.leicabiosystems.com/fileadmin/img_uploads/novocastra_reagents/Novocastra_datasheets/laminin.pdf]
6. Sarangarajan R, Vedam VK, Sivadas G, Krishnaraj R, Sarangarajan A, Shanmugam KT. Pseudoepitheliomatous hyperplasia: Relevance in oral pathology. J Int Oral Health 2015 Jul;7(7):132–6.
7. 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 Aug;129(8):1032–6.
8. Barsky SH, Siegal GP, Jannotta F, Liotta LA. Loss of basement membrane components by invasive tumors but not by their benign counterparts. Lab Invest 1983 Aug;49(2):140–7.
9. Matus DQ, Chang E, Makohon-Moore SC, Hagedorn MA, Chi Q, Sherwood DR. Cell division and targeted cell cycle arrest opens and stabilizes basement membrane gaps. Nat Commun 2014 Jun 13;5:4184.
10. Zaha DC. Significance of immunohistochemistry in breast cancer. World J Clin Oncol 2014 Aug 10;5(3):382–92.
11. Drexler W, Fujimoto GJ. Optical Coherence Tomography: Technology and Applications. (Biological and Medical Physics, Biomedical Engineering). USA: Springer-Verlag Berlin Heidelberg; 2008.

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Written informed consent was obtained from the patient for publication of this study.

Conflict of Interest
Authors declare no conflict of interest.