Detection of Microinvasion in Vulvar and Cervical Intraepithelial Neoplasia Using Double Immunostaining for Cytokeratin and Basement Membrane Components

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• Context.—Identification of early invasion in vulvar intraepithelial neoplasia 3 (VIN 3) and cervical intraepithelial neoplasia 3 (CIN 3) may be difficult with the use of routine hematoxylin-eosin staining. Presence of obscuring inflammation and tangential tissue sectioning are the most common diagnostic pitfalls.

Objective.—To examine the utility of double immunostaining for cytokeratin–collagen IV or cytokeratin–laminin in the detection of early invasion in VIN 3 and CIN 3.

Design.—The study group consisted of 10 cases of “VIN 3, suspicious for invasion” and 10 cases of “CIN 3, suspicious for invasion.” The negative control group consisted of VIN 3 (n = 15) and CIN 3 (n = 10). The positive control group consisted of cases of invasive vulvar carcinoma (n = 11) and invasive cervical carcinoma (n = 25). All cases were double immunostained for cytokeratin and collagen IV and, in a separate reaction, for cytokeratin and laminin. The continuity of the basement membrane and the presence of stromal invasion were assessed in the stained sections.

Results.—The staining for collagen IV and laminin yielded identical results. A well-defined, continuous basement membrane was visualized in all cases of VIN 3 and CIN 3. A discontinuous or absent basement membrane was observed around the malignant cells on the invasive tumor front in all cases of vulvar and cervical carcinoma. In 2 of 10 cases of VIN 3, suspicious for invasion and in 4 of 10 cases of CIN 3, suspicious for invasion definitive foci of microinvasion were identified with the use of double immunostaining. A well-defined, continuous basement membrane was present in the remaining cases “suspicious for invasion.”

Conclusion.—Double immunostaining for cytokeratin–collagen IV or cytokeratin–laminin is useful for evaluation of early invasion in equivocal cases of VIN 3 and CIN 3.

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brane (BM) components as an adjunct to light microscopy in the diagnosis of superficial invasion in various carcinomas. Yet the use of this technique to investigate the presence of BM in in situ and invasive lesions of various tissues has produced conflicting results. Some of the studies have reported areas of discontinuous BM in cases of cervical intraepithelial neoplasia 3 (CIN 3) or carcinoma in situ of the skin,2-4 whereas a well-formed BM was identified in a proportion of invasive cervical and skin carcinomas.2-11 In addition, metastatic foci of squamous cell carcinoma in the lymph nodes were found to be surrounded by BM.7,8,12 Some of the authors concluded that immunohistochemical staining for the elements of BM may be of limited value in cases with questionable stromal invasion.4

Other studies, however, reported the presence of continuous BM in all cases of CIN 3-11. A more precise analysis of BM in carcinoma in situ of the skin revealed that some of the reported breaks were present in the areas of regression of the lesion, where BM was reduplicated and the breaks were seen in the “old” BM.7-9 In addition, diminutive BM breaks were observed in the areas of marked inflammation where mononuclear cells could be seen crossing through BM into the epithelium. However, no migration of the dysplastic epithelial cells in the opposite direction was identified in these cases.7-9 Detailed studies of BM in invasive carcinomas of the cervix and skin2-11 revealed that most tumors retained the capacity for BM synthesis, since collagen IV and laminin were identified surrounding invasive and metastatic tumor nests. Well-differentiated tumors were more likely to show positive staining for BM components, whereas poorly differentiated tumors were more likely to lack staining.5,9,10 Although many tumors were shown to produce BM, definitive BM gaps were described at the invasive tumor fronts or in the areas of microinvasion.7,11,12

As demonstrated in the previous studies, the mere presence or absence of BM components is not sufficient evidence of the presence or absence of invasion. However, a visualization of the dysplastic epithelial cells breaching the BM gaps into the stroma, using double immunostaining for cytokeratin (CK) and BM components, may provide convincing evidence of stromal invasion. Recently, a technique for double immunostaining with CK and smooth muscle actin has been developed in our department for detection of early invasion in breast carcinomas.13 In the current study, we sought to determine if a similar technique, using double labeling for CK and collagen IV or CK and laminin, might be useful in assessing the presence or absence of invasion in problematic cases of CIN and vulvar intraepithelial neoplasia (VIN).

**MATERIALS AND METHODS**

**Case Selection**

The surgical pathology files of the Department of Pathology, Weill Medical College of Cornell University, were searched from 1997 through 2002 to identify successive vulvar and cervical specimens. The study group consisted of 10 cases of VIN 3 with areas suspicious for invasion and 10 cases of CIN 3 with areas suspicious for invasion. The negative control group consisted of 15 cases of VIN 3 and 10 cases of CIN 3. The positive control group consisted of superficially invasive vulvar carcinoma (n = 1), invasive vulvar carcinoma (n = 10), microinvasive cervical squamous cell carcinoma (n = 15), and invasive cervical squamous cell carcinoma (n = 10).

**Immunohistochemical Analysis**

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded 5-μm tissue sections in TechMate automated immunostainer (Ventana Medical Systems Inc, Tucson, Ariz) using anti-laminin monoclonal antibody (clone LAM-90, Novocastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom), anti-collagen IV antibody (clone CIV 22, DakoCytomation, Carpinteria, Calif), and anti-human CK monoclonal antibody (clone MNF 116, DakoCytomation). The immunostaining consisted of 2 steps; in the first step, after antigen retrieval in 0.1% trypsin at 37°C for 25 minutes, the sections were incubated with anti-laminin antibody at a dilution of 1:100 for 1 hour or with anti-collagen IV antibody at a dilution of 1:200 for 1 hour, followed by a horseradish peroxidase-labeled mouse Envision Plus detection system (DakoCytomation) for 30 minutes. Peroxidase reaction was developed using diaminobenzidine liquid chromogen (DakoCytomation), resulting in a dark brown color of the BM. In the second step, sections were incubated with anti-CK (MNF 116) antibody at a dilution of 1:600 for 50 minutes, followed by secondary goat anti-mouse biotinylated antibody, and finally followed by the avidin-biotin complex alkaline-phosphatase detection system (Ventana Medical Systems) with BT Red reagent substrate chromogen resulting in a bright red staining of the epithelium. Sections were then counterstained with hematoxylin and mounted. There is an excess of secondary antibodies in the first step of staining using horseradish peroxidase-labeled mouse Envision Plus detection system, which results in complete saturation of respective epitopes on the primary mouse antibodies, and therefore we did not observe any cross-reactivity between the secondary anti-mouse antibodies used in the second step and the primary mouse antibodies used in the first step of staining. The technical aspects are discussed in detail in the article by Prasad et al.13

**RESULTS**

**Comparison of Double Immunostaining for CK–Collagen IV and CK–Laminin**

The immunostaining for CK using alkaline-phosphatase complex with BT Red reagent substrate resulted in red staining of the epithelium, whereas immunostaining for laminin or collagen IV using diaminobenzidine liquid chromogen resulted in dark brown staining of the BM (Figure 1, A). There were no significant differences between the results of the staining for CK–collagen IV or CK–laminin. The staining for laminin resulted in a visualization of a slightly thicker membrane. Rarely, laminin showed cytoplasmic staining in the basal cells of the normal or dysplastic epithelium. The archival cases stored more than 10 years showed inconsistent staining for both collagen IV and laminin. In cases less than 10 years old, the staining was consistent; however, in few cases the pretreatment digestion time required adjustment because of variation of adequacy of specimen fixation. Long digestion of tissue sections sporadically caused some epithelial cells to become dis cohesive and displaced on the slide; the displaced cells were easily identified as an artifact, because they were in a different plane of focus from the rest of the tissue.

**Double Immunostaining in the Negative Control Cases:**

**VIN 3 and CIN 3**

A well-defined, continuous BM was visualized in all cases of VIN 3 and CIN 3 (Figure 1, B). The staining intensity for collagen IV or laminin in these cases was identical to that of normal cervical and vulvar mucosa (Figure 1, A). In cases with inflammation at the epithelial-stromal junction, inflammatory cells were seen migrating through...
Figure 1. Control cases. Double immunostaining for cytokeratin and collagen IV. A, Normal vulvar epithelium (original magnification ×100). B, Vulvar intraepithelial neoplasia 3 (original magnification ×100). C, Invasive vulvar carcinoma, no basement membrane staining (original magnification ×100). D, Invasive vulvar carcinoma, focal basement membrane staining (original magnification ×100). E, Verrucous carcinoma (original magnification ×100), pushing front with continuous basement membrane staining. F, Verrucous carcinoma (original magnification ×400), single tumor cells below the pushing tumor front. G, Microinvasive cervical carcinoma (original magnification ×40).
the micropores in BM. Small size, scant cytoplasm, and lack of red CK staining clearly identified these cells as nonepithelial. In the areas of marked acute and chronic inflammation, focal duplication of the BM was occasionally observed; however, the continuity of the main membrane was always preserved in these areas. Tangential or parallel sectioning through the BM resulted in an apparent widening of the membrane with slight decrease in the staining intensity.

**Double Immunostaining in the Positive Control Cases: Vulvar and Cervical Carcinoma**

A discontinuous or absent collagen IV and laminin staining was observed around the malignant cells on the invasive tumor front in all cases of vulvar and cervical carcinoma, both invasive and microinvasive. In some cases, the entire tumor failed to stain (Figure 1, C—invasive carcinoma that lacked collagen IV cuff). In most cases, however, focal continuous collagen IV and laminin staining was present, especially around larger, more centrally located tumor nests (Figure 1, D—continuous collagen IV staining around the larger tumor nest and no staining around the smaller tumor nest below). In 2 cases of verrucous carcinoma of the vulva, a well-defined, continuous BM was visualized on the pushing tumor front (Figure 1, E); however, after a thorough examination of multiple tumor sections, small foci of tumor nests and single tumor cells devoid of BM were identified in the stroma below the pushing front (Figure 1, F). The CK stain was particularly helpful in detecting these areas. In some cases of microinvasive carcinoma of the cervix, there was intense inflammatory infiltrate present in the stroma, obscuring the invasive epithelial nests and impeding the assessment of the tumor dimensions. Again, the CK stain was helpful in highlighting the invasive tumor within the inflammatory infiltrate (Figure 1, G), facilitating more accurate measurement of the tumor size.

**Double Immunostaining in Equivocal Cases of CIN and VIN Suspicious for Invasion**

In 2 of 10 cases of “VIN 3, suspicious for invasion” and in 4 of 10 cases of “CIN 3, suspicious for invasion,” definitive foci of microinvasion were identified with the use of double immunostaining, and these cases were reclassified as superficially invasive or microinvasive carcinoma. A well-defined, continuous BM was visualized in the remaining cases suspicious for invasion. Figure 2, A, illustrates a case of CIN 3 suspicious for invasion with a routine hematoxylin-eosin stain. Intense inflammatory infiltrate obscures the epithelial-stromal junction of the rete ridge indicated by the arrowhead. The CK—collagen IV stain (Figure 2, B) of the same area demonstrates no collagen IV staining around the suspicious focus compared with a well-defined, continuous staining around the adjacent rete ridge. Figure 2, C, shows a hematoxylin-eosin stain of a case of VIN 3 suspicious for invasion. The CK—collagen IV stain of the same focus (Figure 2, D) visualized a large gap in the BM, with epithelial cells transgressing into the stroma. Figure 3, A through D, illustrate further examples of microinvasive foci in cases of VIN 3 suspicious for invasion identified with the use of CK—collagen IV immunostain. An unsuspected capillary lymphatic space invasion was identified using the stain (Figure 3, D), with a red-staining squamous cell present in the lumen of the capillary channel. This small tumor cell was interpreted as endothelial on the routine hematoxylin-eosin section.

**COMMENT**

The process of cancer invasion has been extensively studied at the ultrastructural level. The microinvasion in the cervical carcinoma at the level of electron microscopy was described by Kudo et al.14 In areas of invasion, the basal lamina of BM disappeared, and pseudopod-like cytoplasmic protrusions of the cancer cells were seen in a direct contact with the stroma. The cytoplasmic protrusions of the invading cells contained abundant 70- to 90-nm vesicles, some of which were open directly to the extracellular matrix of the stroma. The vesicles were not observed in carcinoma in situ adjacent to intact BM. The authors suggested that the substances contained in the vesicles may play a role in BM destruction. In addition, the cells traversing the BM gaps showed accumulation of actin filaments in the pseudopod protrusions. These local aggregates of cytoskeletal structures, not observed in adjacent carcinoma in situ, were thought to facilitate an amoeba-like movement of the cancer cells. The destruction of the BM with migration of the cancer cells through BM gaps has been observed universally at the invasive tumor fronts in cancers that arise from different epithelial types.15–18 On the other hand, however, BM deposition around tumor nests has been also widely described.2–12 A plausible explanation of these seemingly conflicting observations was proposed by Liotta,19 whose findings were further corroborated by the works of Cam et al.19 and Ehrmann et al.11 It appears that cancer nests proceed through cycles of growth surge with BM destruction and stromal invasion followed by quiescence and BM re-formation. During the quiescent phase, BM remains relatively intact until a new surge of growth, during which BM is focally dissolved and newly formed tumor buds grow out of the old nest. The degree of BM production varies among different tumor types.

The current study is the first, to our knowledge, to describe double immunostaining for CK and BM components in evaluation of microinvasion in CIN and VIN. Since evaluation of BM continuity with the use of single antibody for collagen IV or laminin may not reliably visualize the areas of microinvasion, we have developed a technique of double immunostaining where the addition of CK staining distinctly highlights the areas of epithelial cells that cross the BM boundaries.

The findings reported herein confirm and expand those reported previously using a single antibody for collagen IV or laminin. Collagen IV and laminin was found to be present, discontinuous, or absent around nests of malignant cells in invasive tumors. As reported in previous studies,11,12 our study has found that the larger and more central tumor nests tend to have better formed BM, whereas the nests and the single tumor cells on the invasive front lack BM or show discontinuous staining. In a case of a specific subtype of vulvar carcinoma, a verrucous carcinoma, the BM was almost entirely intact; however, a few small tumor nests and single cells devoid of BM were identified focally below the pushing tumor border. This is in keeping with other studies of similar tumors of the head and neck characterized by a pushing type of border.12 The retention of BM in verrucous carcinoma correlates with relatively good clinical outcome. Verrucous carcinomas are known for their indolent, localized, superficial
growth, devoid of metastatic potential. This observation further confirms that dissolution of BM facilitates destructive stromal invasion and metastatic spread of cancers.

Although several previous studies have described focal discontinuity of the BM in situ lesions,\textsuperscript{2-8} others reported intact BM,\textsuperscript{9-11} and none of the cases of CIN 3 or VIN 3 reviewed in this study showed significant BM disruption. A migration of inflammatory cells through the micropores

Figure 2. Study cases. A, Cervical intraepithelial neoplasia 3 (CIN 3) suspicious for invasion (hematoxylin-eosin, original magnification ×200). B, CIN 3 suspicious for invasion (cytokeratin and collagen IV stain, original magnification ×200). C, Vulvar intraepithelial neoplasia 3 (VIN 3) suspicious for invasion (hematoxylin-eosin, original magnification ×200). D, VIN 3 suspicious for invasion (cytokeratin and collagen IV stain, original magnification ×200). Arrowheads indicate areas of microinvasion.
in BM was evident in the areas of inflammation, but the pores were smaller than the size of the epithelial cells; furthermore, staining for CK did not reveal epithelial cell movement across BM. The process of leukodiapedesis, although similar to that of cancer cell invasion, involves only a focal and limited proteolytic degradation of BM components.\(^1\),\(^2\)

To our knowledge, only one prior study\(^4\) reported the use of collagen IV immunostaining to reevaluate cases in which previous diagnosis, using light microscopy only, had been inconclusive as to the presence or absence of invasion. In that study, 6 of 15 cervical biopsy specimens were reclassified as showing definitive invasion after correlation of the routine sections with the results of immunohistochemical analysis. In our study, 4 of 10 inconclusive cervical biopsy specimens and 2 of 10 inconclusive vulvar biopsy specimens were shown to have areas of definitive invasion. These findings indicate that a definitive diagnosis as to the presence or absence of microinvasion can be achieved in equivocal cases. The use of immunohistochemical analysis to demonstrate the absence of invasion in areas in question can spare the patient and clinician unnecessary procedures and anxiety. Likewise, the confirmation of invasion in problematic cases can facilitate and speed the proper and definitive treatment.

The use of the anti-CK antibody in our study provided some additional useful information. In one of the cases, the use of CK staining allowed identification of a focus of lymphatic invasion not evident on light microscopy, which would have also been missed had only BM staining been used. The CK immunostaining also visualized the invading single tumor cells in verrucous carcinomas in which the BM was almost entirely intact. Finally, CK staining helped to highlight the invasive tumor cells obscured by severe inflammation. Thus, the extent of the invasive component, which is of prognostic significance, may be more accurately assessed in some cases based on CK immunostaining.

Finally, the use of immunohistochemical techniques for the evaluation of invasion is particularly suited to these cases in which minimal invasion is suspected. Our study demonstrates, as have many previous studies that examined the expression of BM by various tumors, that invasive tumors can indeed produce BM. Thus, tumors that have already developed extensive invasion may yet express intact or relatively intact BM around some nests. Fortunately, such cases rarely pose a diagnostic dilemma.

In conclusion, the results of the current and previous studies indicate that invasive tumor is not defined simply by the absence of BM, and the presence of intact BM surrounding tumor is not proof that a lesion is not invasive. Rather, what defines invasion is the ability of tumor cells to traverse the BM, which initially contains them. This is the process manifested in minimally invasive disease, which may be precisely visualized by the technique described herein.

References


Double Immunostaining for CK and BM Components—Rush et al


