Laminin-5 as a Marker of Invasiveness in Cervical Lesions

Barbro Skyldberg, Sirpa Salo, Elina Eriksson, Ulla Aspenblad, Birgitta Moberger, Karl Tryggvason, Gert Auer

Background: Treatment decisions for cervical cancer, a common disease worldwide, depend on demonstrating whether or not tumor invasion of the surrounding tissue has occurred. Invasion can be difficult to assess by standard histopathologic methods, especially when limited amounts of tissue are available. Several studies of a variety of cancers have reported increased expression of laminin-5—an important attachment protein for epithelial cells—in invasive carcinomas. This study was designed to investigate whether the presence of laminin-5 is related to the invasive capacity of cervical lesions. Methods: We used immunohistochemical methods to stain archival, paraffin-embedded sections of cervical lesions with a polyclonal antibody specifically targeting the γ2 chain of human laminin-5 protein. The study sample included 23 lesions of mild and moderate dysplasia (cervical intraepithelial neoplasia [CIN] 1 and 2, respectively), 32 lesions of severe dysplasia or carcinoma in situ (CIN 3), 15 lesions of microinvasive cancer, and 20 lesions of frankly invasive cancer. Cellular proliferative activity was also investigated by the use of monoclonal MIB-1 (directed against the antigen Ki-67) and anticyclin A antibodies. Results: Invasiveness of cervical lesions was positively associated with immunohistochemical staining of the γ2 chain of laminin-5 (two-sided P = .001). All CIN 1 and CIN 2 lesions—except one CIN 2 lesion later shown to be invasive cancer—and 21 CIN 3 lesions tested negative for the γ2 chain of laminin-5. Eleven CIN 3 lesions and all invasive cancers tested positive for this protein. One lymph node metastasis and a pleural metastasis from one of the patients with invasive cancer showed strong immunohistochemical positivity. Proliferative activity increased with advancement of the lesion but was not confined to cells positive for the γ2 chain of laminin-5. Conclusions: These data suggest that antibodies directed against the γ2 chain of laminin-5 can identify cervical lesions with invasive capacity and thus may be useful as a sensitive marker of early invasion. [J Natl Cancer Inst 1999;91:1882–7]

Cancer of the uterine cervix is one of the most frequent malignancies in women worldwide and the most common cancer in developing countries. Approximately 437,000 new cases of cervical cancer are diagnosed each year, and about 200,000 women die of the disease (1). In Sweden, it currently accounts for 2.4% of female cancers, a considerable reduction from 8.4% over the last 20 years (2). This fact is most likely due to the effectiveness of the cervical screening program in Sweden.

Approximately 90% of all cervical malignancies are squamous cell carcinomas. It is well documented that the sexually transmitted, genital human papillomaviruses (HPVs) (about 30 have been identified, mainly consisting of HPV16 and HPV18) are able to transform immature epithelial cells into precancerous cells (3, 4). The precancerous stages develop preferentially from the immature cells in the transformation zone at the junction between the cervical squamous and glandular epithelia (5). The precancerous stages of invasive cervical carcinoma are defined as different grades of dysplasia; mild (cervical intraepithelial neoplasia: CIN 1), moderate (CIN 2), and severe dysplasia or carcinoma in situ (CIN 3) (6). Approximately 30% of all carcinomas in situ, when left untreated, are suggested to develop into invasive cancer after 13 years or longer (7). A later investigation (8) reported the proportion of cases of new carcinoma in situ that progressed to invasive cancer to be 12.2%, with a mean duration of the in situ stage of 13.3 years.

Treatment of cervical lesions is totally dependent on histopathologic judgment of whether or not a lesion is invasive. This distinction can be extremely difficult to assess, especially in small biopsy specimens and curettage material. A sensitive and objective diagnostic procedure determining the invasive potential of cervical neoplastic cells would, therefore, be of substantial value.

Invasion of the cancer into the stromal tissue requires, first, the ability of the cells to penetrate the underlying basement membranes and, second, migration that involves adhesion to extracellular matrix constituents, such as laminins, collagens, and fibronectins. Laminins are a family of extracellular proteins that constitute a major component of basement membranes. The laminin molecules are heterotrimeric proteins formed by the association of three different gene products, one heavy α chain and two light β and γ chains [nomenclature according to Burgeson et al. (9)]. To date, five α chains, three β chains, and three γ chains that are known to form at least 10 laminin isoforms have been reported (10–13). Laminin-5, previously termed kalinin, nicein, epiligrin, or ladisin, consists of α3 (14), β3 (15), and γ2 (16) chains. It is intimately involved in the attachment of epithelial cells such as keratinocytes to the basement membranes (17–22). Accumulating data suggest increased laminin-5 γ2 chain expression in most cases of carcinomas studied so far, i.e., colorectal, pancreatic, and oral cancers (23–27), but decreases have also been reported—e.g., in prostate cancer (28). In normal epithelial cells, the expression of laminin-5 is strongly related to tissue renewal. During healing skin wounds, strong laminin-5 expression has been observed in migrating keratinocytes (14, 23, 29). In 1995, Pyke et al. (24) suggested that laminin γ2 chain expression can serve as a marker of invasive cancer in colon adenocarcinomas and in various types of squamous cell carcinomas. Similar results were reported by Sordat et al.

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See “Notes” following “References.”

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(27) for colorectal carcinomas. Laminin-5 has previously been shown to be a ligand for the integrins α3β1, α6β1, α6β4, and α2β1 (18, 30–32). Sordat et al. (27) showed a decrease in the level of α6β4 integrin in colorectal cancer, together with increased laminin-5 expression.

In this study, we have evaluated expression of the γ2 chain of laminin-5 as a potential marker for early invasiveness in clinical lesions. Analyses of 90 lesions varying in neoplastic advancement from mild dysplasia to invasive carcinoma demonstrated association of expression of the γ2 chain with invasive potential.

**MATERIALS AND METHODS**

**Tissue Samples**

Stored biopsy material from 14 women with cervical lesions diagnosed at the Department of Pathology, Sabbatsberg Hospital, Stockholm, Sweden, from 1970 through 1977 and in 1992 and from 47 women diagnosed at the Department of Pathology, Karolinska Hospital, Stockholm, Sweden, from 1992 through 1998 was used in this study. The study protocol was cleared by the ethical committee of the Stockholm County Council. The biopsy material was formaldehyde fixed, paraffin embedded, and diagnosed on hematoxylin–eosin (H–E)-stained tissue sections. The evaluated material consisted of four lesions of mild dysplasia (CIN 1), 19 lesions of moderate dysplasia (CIN 2), 32 lesions of severe dysplasia or carcinoma in situ (CIN 3), 15 lesions of microinvasive cancer, and 20 lesions of frankly invasive cancer. For some women with multiple diagnoses (e.g., coexisting CIN 1, CIN 3, and microinvasive cancer), specimens of lymph node metastases in the pelvic wall and metastases in the pleura were studied. From each specimen, four consecutive sections (4 μm thick) were cut and put onto specially treated slides (Menzel Superfrust plus) for immunohistochemical studies of the γ2 chain of laminin-5, Ki-67, and cyclin A. Sections for H–E staining were cut before and after the other sections to confirm the diagnosis. All cases were selected by original histopathologists (E. Eriksson and G. Auer) and translated into the Richart (6) grading system according to the procedure of Michael (33) for immunohistochemical staining was performed. Invasiveness was graded according to the International Federation of Gynecology and Obstetrics (FIGO) classification system (33) as follows: cancer in situ (FIGO 0), microinvasive cancer (FIGO IA), and frankly invasive cancer (FIGO IB).

**Immunohistochemistry**

Preparation and characterization of polyclonal antibodies raised in rabbit against a fusion protein containing the C terminus of the laminin γ2 chain (containing amino acid residues, Nos. 1017–1178) and glutathione S-transferase were performed according to methods described earlier (34).

Immunohistochemistry was performed by use of the standard horseradish peroxidase avidin–biotin complex (ABC) technique (Elite Standard Kit, cat. PK-6100; Vector Laboratories, Inc., Burlingame, CA). The sections were first deparaffinized, rehydrated, and microwave treated in 0.01 M sodium citrate buffer (pH 6) for 10 minutes at 500 W. After the sections were rinsed in Tris-buffered saline (TBS) (pH 7.6), the endogenous peroxidase activity was blocked by immersion of the slides in 0.5% hydrogen peroxide in distilled water for 30 minutes and unspecific staining was prevented by use of 1% bovine serum albumin (BSA) in TBS for 20 minutes. After incubation overnight at 4 °C with the rabbit γ2 chain antibodies diluted 1:200 in 1% BSA (~2 μg/mL), a biotinylated antirabbit immunoglobulin G (diluted 1:200) was applied for 30 minutes, followed by incubation in the avidin–biotin–peroxidase complex for 30 minutes. The peroxidase reaction was developed by use of diaminobenzidine tetrahydrochloride (0.6 mg/mL with 0.03% H2O2 for 6 minutes. TBS was used for rinsing between steps. After counterstaining with Mayers’ hematoxylin, the slides were dehydrated and mounted with a xylene-soluble mounting medium. As a control of specificity of the method, the laminin γ2 chain antibody was replaced with BSA, and the same procedure was performed on adjacent sections. Only cells with a distinct cytoplasmic immunoreaction were considered laminin-5 γ2 chain positive. To declare a lesion positive, more than 1% of the cells had to show this specific immunostaining.

The Ki-67 antigen, a proliferation-associated nuclear protein, was detected by the monoclonal mouse antibody MIB-1 (Immumotech S.A., Marseille, France; diluted 1:150 in 1% BSA), and cyclin A analysis was performed with a monoclonal mouse antibody to human cyclin A protein (Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.; diluted 1:100 in 1% BSA). The MIB-1 antibody allows discrimination between nonproliferating cells and proliferating cells in all phases of the cell cycle, whereas the cyclin A antibody targets the committed cells—i.e., cells that are committed to completing the cell cycle.

The evaluation of the immunohistochemical reactions was done by two investigators working independently of each other.

**Statistical Analysis**

A chi-squared test for trend (35) was used to analyze the association between positivity for laminin-5 γ2 chain staining and invasiveness of cervical lesions. A two-sided P value was calculated on the basis of the chi-squared test. Since there were only four lesions with CIN 1, CIN 1 and CIN 2 were grouped together.

**RESULTS**

Fig. 1 summarizes the results of laminin γ2 chain immunoreactivity in 90 paraffin-embedded lesions from the uterine cervix. Only one (see below) of 23 CIN 1 and CIN 2 lesions tested positive for the laminin-5 γ2 chain by use of a polyclonal antibody. Twenty-one of 32 CIN 3 lesions were negative for laminin-5 γ2 (Fig. 2, A and B). All lesions with invasive and microinvasive cancers (total, 35 lesions) were positive.

On treatment with MIB-1 antibodies to Ki-67 and antibodies to cyclin A, histologic sections of CIN 1 and CIN 2 lesions showed proliferative activity in the parabasal cell layers, with no activity in the basal cells. Cells that were positive for the laminin γ2 chain in the CIN 3-diagnosed material were in close conjunction with the basement membrane. The staining was weak to moderate and cytoplasmic.
In some CIN 3 specimens, positive cells in the epidermal layer overlying the lesion were visible that were probably participating in wound healing. In the CIN 3 lesions, proliferative activity was observed mainly in the parabasal and intermediate layers. The degree of proliferative activity was independent of the laminin $\gamma 2$ chain positivity.

In the specimens with microinvasive and frankly invasive cancers, immunohistochemical staining of laminin $\gamma 2$ chain was mainly confined to the cancer cells at the invasion front of the tumor. The staining was moderate to strong and exclusively cytoplasmic (Fig. 2, E–H). Proliferative activity was seen throughout the tumor areas but had no specific association with the peripheral $\gamma 2$ chain-positive cancer cells.

By use of a variation of the chi-squared test (35), a statistically significant association between grade or degree of invasiveness of cervical lesions and laminin $\gamma 2$ positivity was observed (two-sided $P<.001$; see Table 1).

One of the lesions diagnosed as CIN 2 tested strongly positive for the $\gamma 2$ chain. Most of the positive cells were strongly stained and peripherally located (Fig. 3, B). Morphologically, the specimen consisted of fragments of strongly chronically inflamed mucosa from the portio tissue of the cervix, covered with inverting metaplastic and degenerative squamous epithelia. The interpretation of the histopathologic picture of the epithelium was difficult because of massive presence of inflammatory cells and was diagnosed as only moderate squamous cell atypia (Fig. 3, A). However, about 1 month later, a cervical cone biopsy from the same patient was diagnosed as microinvasive cancer. This later specimen showed a strong cytoplasmic staining for the laminin $\gamma 2$ chain and also a tendency to extracellular staining (Fig. 3, C and D).

Sections from samples of a local lymph node metastasis and a pleural metastasis from the same patient are shown in Fig. 3, E–H. The lymph nodes demonstrated a small number of $\gamma 2$ chain-positive cancer cells (Fig. 3, F), while the pleural metastasis showed peripherally situated cancer cells with strong cytoplasmic laminin $\gamma 2$ positivity (Fig. 3, G and H). Proliferative activity was shown throughout the metastases by the strong positivity for both the MIB-1 and cyclin A antibodies.

**DISCUSSION**

In this study, we used a polyclonal antibody against the recombinant $\gamma 2$ chain of laminin-5 to examine the location of this protein among 90 lesions comprising both precancerous stages and frank cancer of the uterine cervix. The goal was to identify a marker of early invasiveness and possibly identify the CIN 3 lesions that were at increased risk of progressing to invasive carcinoma. Pyke et al. (24) demonstrated by immunohistochemistry, (Fig. 2, C and D). In some CIN 3 specimens, positive cells in the epidermal layer overlying the lesion were visible that were probably participating in wound healing. In the CIN 3 lesions, proliferative activity was observed mainly in the parabasal and intermediate layers. The degree of proliferative activity was independent of the laminin $\gamma 2$ chain positivity.

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![Fig. 2. Lesions of the uterine cervix. Carcinoma in situ: hematoxylin–eosin (H–E) staining (A); laminin $\gamma 2$ chain immunohistochemical staining (B). Basal and parabasal cells are negative for the staining by the antibody to the laminin $\gamma 2$ chain (B). Carcinoma in situ: H–E staining (C); laminin $\gamma 2$ chain immunohistochemical staining (D). Basal and parabasal cells show weak to moderate cytoplasmic staining (D). Microinvasive cancer: H–E staining (E); laminin $\gamma 2$ chain immunohistochemical staining (F); and invasive cancer laminin $\gamma 2$ chain immunohistochemical staining (G and H). Moderate to strong cytoplasmic staining, with all staining confined to the cancer cells at the invasion front of the tumor in both the microinvasive and invasive cancers (F, G, and H). Original magnifications were ×100 for A–D, ×33 for E–G, and ×66 for H.](image-url)

**Table 1. Association between positivity for laminin $\gamma 2$ staining and invasiveness of cervical lesions*"**

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Total No.</th>
<th>No. laminin positive</th>
<th>%</th>
<th>$\chi^2$†</th>
<th>$P$, two-sided</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild + moderate dysplasia (CIN 1, 2)</td>
<td>23</td>
<td>1</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma in situ (CIN 3)</td>
<td>32</td>
<td>11</td>
<td>34.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microinvasive carcinoma</td>
<td>15</td>
<td>15</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>20</td>
<td>20</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>52.3</td>
<td>&lt;.001</td>
<td></td>
</tr>
</tbody>
</table>

* CIN = cervical intraepithelial neoplasia.
† Chi-squared for trend, 1 df.
by use of a polyclonal antibody, the localization of laminin γ2 chain protein in cancer cells in all cases studied of colon adenocarcinomas and squamous cell carcinomas of the skin and cervix but not in sarcomas. They also showed that, by in situ hybridization, the distribution of laminin-5-positive cancer cells at the invasion front in colon carcinomas was identical to that of the receptor for urokinase plasminogen activator (uPAR). An earlier study by Pyke et al. (36) proposed that binding of the ligand for uPAR promotes the cancer cell invasion process by activation of plasminogen, leading to degradation of extracellular matrix. The coexpression of the γ2 chain of laminin-5 and uPAR suggested laminin-5 as a marker of invasive cancer in some human cancers (24). Sordat et al. (27) (studying colorectal neoplasia) and Pyke et al. (24) (studying colon carcinoma) observed budding cancer cells with the accumulation of laminin-5 γ2 chains in the cytoplasm. In contrast, extracellular γ2 expression has been reported in gastric cancer in basement membranes surrounding cancer cells (37).

The high incidence of cervical cancer worldwide, almost half a million a year, with a mortality of about 50%, constitutes a major public health problem (1). With regard to diagnosis, differentiation between CIN 3, microinvasive, and frankly invasive cancer is an important and difficult question to answer in some cases. Microinvasive cancer is defined according to the FIGO classification system (33) as a lesion no wider than 7 mm in which neoplastic epithelium invades the stroma in one or more places to a depth of 5 mm or less below the basement membrane of the epithelium. The frequent occurrence of inflammation in the endocervix often makes it difficult to determine whether the diagnosis is CIN 3 (FIGO 0) or microinvasive cancer (FIGO IA), since the border of the epithelia is often uneven or indistinct. Depending on the diagnosis, the patient will receive different treatments. With a diagnosis of CIN 3, a laser treatment or conization will be performed followed by regular controls, in Sweden first every 6 months up to 2 years and then once a year up to 5 years. If microinvasive cancer is diagnosed, the treatment will be a simple hysterectomy. In contrast, frankly invasive cancer (FIGO IB) is treated with radical hysterectomy and bilateral pelvic lymphadenectomy. Thus, a sensitive diagnostic procedure is a prerequisite for appropriate therapy. According to the classic works of Petersen (7) and Kottmeier (38), 30%–70% of all cervical carcinoma in situ lesions had progressed to invasive carcinoma after 10 years of observation. Mean intervals between the time of detection of carcinoma in situ and invasive carcinoma ranged from 8 to 20 years in different studies. The latency period can vary with age, and progression may be more rapid in elderly women than in younger women (39). A 40-year study of repeated screening of a younger and an older age group of women estimated regression rates for carcinoma in situ to be 72% and 47%, respectively (40).

In our study, all microinvasive and frankly invasive cancers showed laminin γ2 chain positivity, with the immunoreactivity located almost exclusively in the cytoplasm of the cancer cells at the invasive front of the cancer, in agreement with the findings of Pyke et al. (24) and Sordat et al. (27). In 11 of 32 lesions with CIN 3, cytoplasmic laminin γ2 chain positivity was demonstrated in the cells close to the basement membrane. All mildly and moderately dysplastic lesions (CIN 1 and 2) were immunohistochemically negative for laminin γ2, with the important exception of the one CIN 2 lesion that 1 month thereafter proved to be microinvasive cancer, which was originally difficult to diagnose because of the presence of inflammatory cells. By means of hematoxylin staining, the invasive cells could easily have been overlooked, but they were clearly identifiable by the laminin γ2 staining.

In contrast to other reports (18,24), we detected no laminin γ2 chain immunoreactivity in the basement membrane of
normal cervical tissue adjacent to carcinomaous areas with our method. A possible explanation could be that the antibody has difficulty reaching the highly cross-linked laminin-5 protein in the native basement membrane.

The investigation of our material with the proliferation markers Ki-67, designed to allow discrimination between nonproliferating and proliferating cells, and cyclin A, which identified committed cells, clearly showed that proliferative activity increased with increasing advancement of CIN 3 and invasive cervical lesions. In both types of lesions, the degree of proliferative activity was independent of laminin γ2 positivity. In the invasive cancers showing a lower degree of differentiation, the proliferative activity was detected throughout the tumor sections but showed no spatial association with the laminin γ2-positive cancer cells located peripherally.

Because of the diagnostic problems in distinguishing between CIN 3 and microinvasive cancer in cervical lesions, there will often be an overtreatment or undertreatment of the patients, which may cause extensive suffering for the patients and high costs for the health care system. It is obvious that there is need for a marker that can identify early the CIN 3 lesions that are likely to progress and develop into invasive carcinomas. This study has shown that our immunohistochemical method, by use of a polyclonal antibody against the recombinant γ2 chain of laminin-5, is able to distinguish between lesions with and without invasive capacity. This method could be useful in the histopathologic diagnosis of cervical cancer.

REFERENCES


NOTES

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