

ORIGINAL ARTICLE

p16^{INK4a} and laminin-5 γ 2 chain expression during the progression of cervical neoplasia

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Abstract

p16^{INK4a}, laminin-5 γ 2 chain, and PCNA were investigated to compare the expression levels in relation to histological diagnosis and time for progression. The material consisted of 37 normal cervical tissues, 35 with different grades of CIN, and 11 invasive cervical cancers. Our results showed a reduction of basement membrane staining for laminin-5 γ 2 chain from 78.4% in normal squamous epithelium to 27.8% in CIN3 ($p < 0.001$). The intracytoplasmic staining for laminin-5 γ 2 chain increases with severity of lesion. The same trend was observed with p16^{INK4a} and PCNA expression ($p < 0.001$). Co-expression of p16^{INK4a} and PCNA was seen in 85.7% of samples. Cases that were laminin-5 γ 2 chain BM –/p16^{INK4a} +/PCNA+ have the shortest interval time (average: 46.8 ± 36.3 months) for progression, while cases with laminin-5 γ 2 chain BM +/p16^{INK4a} –/PCNA – have the longest time interval (average: 110.2 ± 52.7 months) ($p < 0.05$). Thus co-expression of p16^{INK4a}, laminin-5 γ 2 chain and PCNA may be valuable for the prediction progression of cervical neoplasia.

Epidemiological studies as well as experimental studies indicated that infection with the human papillomavirus (HPV) is the most important etiological factor in the development of cervical cancer [1,2]. A series of different types of HPV has been associated with diseases of the genital tract mucosa. Of these, HPV 16, 18, 45, 31 and 33 are the most prevalent among cervical carcinomas [3,4]. HPV DNA is present in about 95% of squamous cell carcinomas [4], while in adenocarcinomas of the cervix, it has been detected in only about 70% to 90% [5,6]. Due to population-based Pap smear screening, the incidence of squamous cell carcinoma has decreased remarkably in most developed and in some developing countries, while during the same period, there has been a relative increase in the incidence of adenocarcinoma among women [7–9].

Several studies have reported this escalation, especially in young women [10]. Cytological screening is apparently not effective in preventing the development of adenocarcinomas [8]. The precancerous stages of invasive cervical carcinoma are defined as different grades of dysplasia. It is suggested that approximately 12% of all carcinomas in-situ, when left untreated, evolved into invasive cancer after at least 13 years. The natural history of cervical intraepithelial neoplasia (CIN) is extremely variable. If left untreated, CIN may regress to normal, persist or eventually progress to invasive cervical cancer. The study of biomarkers that can distinguish CIN cases that will progress or regress has significant clinical value.

The conditions required for invasion of the cancer into the stromal tissue are, first, the ability of the

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cells to penetrate the underlying basement membranes and second, migration that involves adhesion to extracellular matrix constituents, such as laminins, collagens, and fibronectins. Laminins regulate cell adhesion, migration and differentiation, and is a family of glycoproteins of the extracellular matrix that constitute a major component of basement membranes. Laminin-5 is one of the isoforms of the laminin family and is composed of α 3, β 3 and γ 2 chains, of which the γ 2 is specific to laminin-5 [11]. Decreased expression of the laminin-5 γ 2 chain has been found in different human cancer tissues such as colon adenocarcinoma, ductal mammary carcinoma, squamous cell carcinoma of the skin, vulva, lung carcinoma, pancreatic adenocarcinoma, breast carcinoma, oral squamous cell carcinoma and tongue squamous cell carcinoma [12,13]. Hao J et al. reported the decreases of expression of laminin-5 γ 2 in prostate cancer [14]. Data from different groups suggested that laminin-5 γ 2 chain expression could be as a marker of invasive cancer in colon adenocarcinomas and in various types of squamous cell carcinomas [12,15]. A correlation of laminin-5 γ 2 chain expression in the cytoplasm and/or the base membrane of cervical epithelium and with the grade of cervical dysplastic lesion has been reported earlier [16]. It was also reported that laminin-5 γ 2 chain could be regarded as a marker for invasiveness of precancerous lesions in the lower anogenital tract [17]. Skyldberg et al. suggested that immunohistochemically determined expression of laminin-5 γ 2 chain could be used as a sensitive marker to discriminate between non-invasive and invasive lesions [18]. Hellman et al. showed that in patients with vaginal cancer, a high expression of the γ 2 chain was significantly correlated with a short-term survival rate [19].

p16^{INK4a}, a tumor suppressor gene, is an element of the p16^{INK4a}/cyclin D-cdk4/6/Rb pathway. It can induce G1 cell cycle arrest by inhibiting the phosphorylation of pRb by cdk4 and cdk6. Recent studies have shown that immunohistochemical staining with p16^{INK4a} is a promising marker for dysplastic and malignant cervical epithelia [20,21]. Klaes et al. found that overexpression of p16^{INK4a} assists in the identification of high-risk HPV-related cervical squamous lesions [20], and Sano et al. found that overexpression of p16^{INK4a} was associated with HPV infection [21,22]. In these studies it was suggested that p16^{INK4a} appears to be useful for distinguishing immature squamous metaplasia from high-grade squamous intraepithelial lesion, where the former could pose as a morphologic differential diagnosis.

Proliferating cell nuclear antigens (PCNA) relates to DNA replication specifically detecting cells with active DNA replication, and it is universally used for

evaluation of cell proliferation by immunohistochemistry. It appears PCNA is a good marker for proliferating cells regardless of the oncogenic capacity of the cells, as it does not distinguish dysplastic proliferating cells from normal proliferating cells. The aims of this study were 1) to investigate the expression of laminin-5 γ 2 chain, p16^{INK4a} and PCNA in serial biopsies with histological diagnosis of different cervical lesions, 2) to evaluate the clinical significance of these proteins during the progression of cervical neoplasm.

Materials and methods

Cases selection

The material was obtained from women diagnosed on histology with CIN3 or invasive cervical cancer who received treatment in Umeå University Hospital. These patients were identified through the pathological registry between the years 1982 to 2000. The inclusion criterion was they were diagnosed with CIN3 or invasive cervical cancer and they have had previous biopsies taken representing different histological diagnosis of CIN. Thus, each case constitutes a series of 2 to 3 consecutive biopsy samples that represent normal squamous epithelium, CIN or invasive squamous cell carcinoma. In a histological section, tissue representing normal epithelium, different grades of CIN or invasive cancer can be present close to or adjacent to each other. In total in the entire material, there were 37 normal squamous cervical epithelium, 35 CIN lesions of different grades and 11 SCC of the cervix. These 15 cases with consecutive biopsies were used to study the importance of biomarkers in a human model for cervical cancer progression. The mean age of the 15 cases with CIN 3 and cancer diagnosis was 42.4 years (range: 24–58). The diagnosis of all cases had been reviewed and reconfirmed by two pathologists. Ethical approval to use the material was obtained for the study.

Immunohistochemistry

All specimens were formalin-fixed and paraffin-embedded. Serial sections (4 μ m thick) were cut and the end section was stained with H & E (haematoxylin and eosin) to ensure that the lesion was still present in the serial sections. The sections were processed for immunohistochemical staining. Sections were immersed in xylene to remove the paraffin followed by rehydration through graded alcohol. Epitope retrieval was performed by heating the sections for 10 minutes in 10 mM citrate buffer (pH 6.0) in a microwave oven. The sections were incubated in 0.75% hydrogen peroxide (H₂O₂) in

methanol for a further 10 minutes to block all endogenous activity. This was followed by blocking of nonspecific binding of primary antibodies to epitopes by a preincubation step with 5% normal goat serum for 30 minutes at room temperature. The primary antibodies were then added and incubated at 4°C overnight. After washing thoroughly with PBS, the sections were incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for 30 minutes at room temperature and developed with AEC+substrate-chromogen (DAKO Cytomation, Glostrup, Denmark). The sections were counterstained lightly with haematoxylin. Tissue sections containing colon cancer were used as positive controls for p16^{INK4a} staining and tissue sections of squamous cell cancer of the lung were used as positive controls for PCNA. Negative controls consist of PBS pH 7.4 in place of primary antibodies.

Preparation and characterization of polyclonal antibodies raised in rabbit against a fusion protein containing the C terminus of the laminin-5γ2 chain (containing amino acid residues # 1017–1178) and GST were performed according to methods described earlier [23,18]. Mouse monoclonal F-12 anti-p16^{INK4a} (Santa Cruz Biotechnology, CA, USA) and mouse monoclonal PC-10 anti-PCNA (Santa Cruz Biotechnology, CA, USA) were diluted 1:100. The secondary antibody was a goat anti-mouse diluted to 1:100 (Amersham Biosciences AB, Uppsala, Sweden). Nuclear staining was considered positive for p16^{INK4a} and PCNA, while cells with distinct cytoplasmic immunoreaction were scored as laminin-5γ2 chain positive. Image analysis was done as described previously [24]. Scoring of immunohistochemistry results were performed on the basis of both of the staining intensity and percentage of immunoreactive epithelial cells in the total of normal or neoplastic cells, as previously described [25,26]. Staining score criteria for p16^{INK4a} and PCNA was as following showed in percent (%):-(-negative): (no expression); <20: weak staining ±; 20–30, weak or moderate staining +; 31–50 moderate or strong staining ++; >50 strong staining +++. The scores +, ++ and +++ were considered positive sample for p16^{INK4a} and PCNA [25–28]. The percentage of Laminin-5γ2 positive cells was estimated and categorized as follows and showed in percent:-(-negative): no expression or <1%, +1–5%, ++ 6–30% and +++ >30%.

Statistical analysis

The data was analyzed with the SPSS program. Statistical analysis was based on χ^2 test with Yates' correction. An additional two-tailed Fisher's exact

test was used only when the number of samples was less than ≤ 5 . The t-test was used to analyze quantitative data. It was considered significant when $p < 0.05$.

Results

Positive expression rates of p16^{INK4a}, laminin-5γ2 and PCNA among different cervical lesions

The expression of p16^{INK4a}, laminin-5γ2 and PCNA were analyzed in 37 normal epithelium of the cervix, 17 CIN1/CIN2, 18 CIN3 and 11 invasive SCC in the biopsies of 15 women. The results of the immunohistochemical staining of p16^{INK4a}, laminin-5γ2 and PCNA of these cases were as summarized in Table I. The frequency of laminin-5γ2 chain staining seen at the basement membrane (BM+) was found in 78.4% (29/37) of normal epithelium, in 41.2% (7/17) of CIN1/CIN2 and in 27.8% (5/18) of CIN3 ($p < 0.001$) (Figure 1A). p16^{INK4a}, the positive rate was significantly higher and it also increases with severity of lesion; CIN1/CIN2 has 64.7% (11/17) of positively stained cells, CIN3 77.8% (14/18) and SCC 86.2% (25/29) as compared to 29.7% (11/37) in normal squamous epithelium ($p < 0.001$). However, the difference between the various histological diagnoses, CIN1/CIN2, CIN3 and invasive cancer was not significant for p16^{INK4a} expression ($p > 0.05$). As for laminin-5γ2 chain expression at the basement membrane of cancerous lesions was negative. No expression was evident in the stromal cells. In comparison, the cytoplasmic staining of laminin-5γ2 was detected in 2.7% (1/37) of normal epithelium, 17.7% (3/17) of CIN1/CIN2 ($p < 0.05$), 31.6% (6/18) of CIN3 ($p < 0.01$), and 89.7% (26/29) in invasive cancers ($p < 0.001$) (Figure 1A). PCNA expression was found in 10.8% (4/37) of normal epithelium, in 35% (6/17) of CIN1/CIN2, 72.2% (13/18) of CIN3 and 93.1% (27/29) of SCC. The rate of PCNA expression between the various histological diagnoses was significantly increased and it increase with the severity of the disease ($p < 0.001$) (Figure 1B).

Expression and co-expression pattern of p16^{INK4a}, laminin-5γ2 and PCNA in the cells of different cervical lesions

Laminin-5γ2 chain expression can be either intra- or extracellular. None of the cells in the normal epithelium expressed any intracytoplasmic laminin-5γ2 chain. Instead, laminin-5γ2 chain was exclusively extracellular in the basement membrane of normal epithelium. With increasing severity of dysplasia, laminin-5γ2 chain expression tends to be more intracellular in the cytoplasm of abnormal cells

Table I. Expression of Laminin-5γ2, p16^{INK4a} and PCNA in the progression of cervical neoplasia

| Lab No. | Case No. | Age (year) | Diagnosis | Sample taking | Laminin-5γ2 | | p16 ^{INK4A} | PCNA |
|---------|----------|------------|-----------|---------------|-------------|-----------|----------------------|------|
| | | | | | BM* | Cytoplasm | | |
| 18a | 1a | 35 | Normal | 1/10/1990 | - | - | + | + |
| b | 1b | 37 | CIN2 | 12/10/1992 | - | - | +++ | +++ |
| 12a | 2a | 47 | Normal | 11/18/1988 | + | - | - | - |
| b | 2b | 53 | CIN3 | 9/5/1994 | - | - | + | ± |
| 4a | 3a | 37 | CIN2 | 91-02-29 | - | - | + | + |
| b | 3b | 42 | Normal | 6/11/1997 | + | - | ± | ± |
| c | 3c | 44 | CIN3 | 9/23/1999 | - | - | ++ | ++ |
| 8a | 4a | 53 | Normal | 11/26/1991 | + | - | + | - |
| b | 4b | 58 | SCC | 6/2/1997 | - | + | - | ++ |
| 2a | 5a | 34 | Normal | 10/24/1990 | + | - | + | - |
| b | 5b | 39 | SCC | 11/29/1995 | - | ++ | ++ | + |
| 5a | 6a | 21 | Normal | 2/10/1986 | + | - | - | - |
| c | 6b | 32 | SCC | 4/27/1997 | - | + | - | - |
| 11a | 7a | 40 | CIN2 | 2/21/1983 | - | - | ++ | + |
| c | 7b | 40 | Normal | 5/20/1986 | + | - | - | - |
| d | 7c | 43 | SCC | 10/19/2000 | - | ++ | ++ | +++ |
| 1a | 8a | 29 | CIN1/2 | 8/16/1982 | - | - | + | - |
| c | 8b | 43 | SCC | 96-09-05 | - | + | ++ | ++ |
| 3a | 9a | 48 | CIN1/2 | 2/15/1985 | + | - | - | - |
| b | 9b | 58 | CIN3 | 11/29/1995 | + | - | ± | +++ |
| 6a | 10a | 39 | Normal | 11/19/1985 | + | - | - | - |
| e | 10b | 50 | SCC | 4/19/1997 | - | + | - | +++ |
| 7a | 11a | 31 | CIN3 | 11/17/1992 | - | - | + | + |
| b | 11b | 35 | SCC | 7/6/1997 | - | + | + | +++ |
| 9a | 12a | 33 | CIN3 | 7/17/1984 | - | - | ++ | ++ |
| c | 12b | 44 | SCC | 5/29/1995 | - | + | ++ | +++ |
| 10a | 13a | 32 | CIN3 | 3/21/1996 | - | - | + | + |
| b | 13b | 34 | SCC | 1/28/1998 | - | + | +++ | ++ |
| 13b | 14a | 23 | CIN3 | 8/6/1993 | + | - | ++ | ++ |
| c | 14b | 24 | SCC | 10/12/1994 | - | - | +++ | +++ |
| 17a | 15a | 41 | CIN2 | 7/10/1992 | + | - | + | + |
| b | 15b | 42 | CIN3 | 1/22/1993 | + | + | +++ | ++ |
| c | 15c | 42 | SCC | 11/17/1993 | - | + | +++ | +++ |

* BM-basement membrane, ±-weak positive.

while its expression at the basement membrane gradually disappears. In tumor cells, laminin-5γ2 chain immunoreactivity was exclusively intracytoplasmic and most of these cells stain moderate to strong. However, the staining intensity appears to decrease to low or to moderate in some of the high-grade CIN lesions. The positively stained cells of high grade CIN were restricted to those projecting into the stroma, and in SCC, the positive cells line the invasion front of the lesion. (Figure 2A, B, C). Unlike laminin-5γ2 chain expression, the expression of p16^{INK4a} was predominately nuclear and it stained cells located in the basal layer of normal epithelium, or in the lower 1/3 layer of CIN1/CIN2, or the entire epithelial layer of CIN3. In SCC, p16^{INK4a} expression was evident in cells occupying the entire lesion invading into the stroma (Figure 2D, E, F). In normal epithelium, few cells stained positive for PCNA and they were located primarily in the basal layer. In CIN1/CIN2, the expression of PCNA was restricted in the lower 1/3 layer to the

upper 1/3 layer while in high grade CIN diagnosis PCNA was present in the entire thickness of the epithelium. Similarly for SCC, the positive cells were seen either at the outer edge of the lesion or they occupy the entire area of the invading front of the tumor (Figure 2G, H, I).

We also looked at the co-expression of p16^{INK4a}, laminin-5γ2 chain and PCNA in the epithelia of these biopsies. Laminin-5γ2 chain staining was primarily positive extracellularly in the basement membrane, and its staining intensity decreases from strong to weak, or disappear with increasing disease severity. p16^{INK4a} and PCNA expression were both nuclear and they were either negative or sporadically positive in the basal layer of normal epithelium in different biopsies (Figure 3A, B, C). In progressing lesions, the staining intensity and the number of p16^{INK4a} and PCNA positive cells were also increased except in some cancer cases where the staining pattern were variable within the lesion (Figure 3D, E, F). Cytoplasmic laminin-5γ2 chain

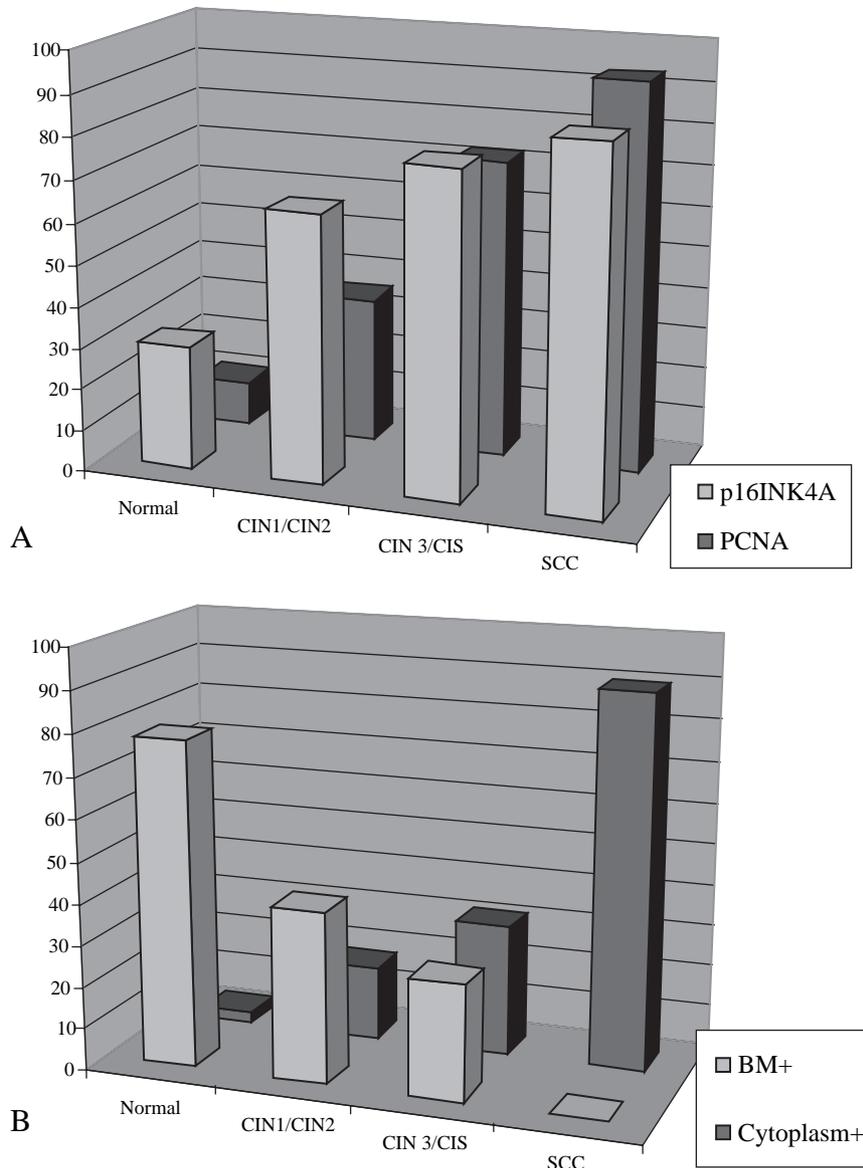


Figure 1. A. The frequency of laminin-5γ2 chain staining seen at basement membrane and cytoplasm of normal epithelium, different stages of CIN and invasive cervical cancer. B. The frequency of p16^{INK4a}, PCNA expression in samples from normal epithelium, different CIN stages, and invasive cervical cancer.

positive cells in SCC were located more at the front or tip of the cancer lesion where as p16^{INK4a} expression was weakly positive and PCNA was negative in the same area (Figure 3G, H, I).

p16^{INK4a}, laminin-5γ2 chain and PCNA expression during progression of cervical neoplasia

The significance the time interval taken for progression from normal or low grade CIN to high grade CIN or SCC in relation to the expression of laminin-5γ2 chain, p16^{INK4a} and PCNA was evaluated. Eight of these cases progressed from normal to CIN or SCC, one case progressed from CIN1/CIN2 to CIN3 and six cases progressed from CIN to SCC.

The expression of these proteins and patient's age, diagnosis and the time the biopsy was taken were shown in Table I. Basement membrane (BM+) staining for laminin-5γ2 chain was 69.6% (7/8) for normal epithelium and 37.5% (6/16) in CIN while the intracytoplasmic staining for laminin-5γ2 chain was absent in (0/8), 12.5% (2/16) and 90.9% (10/11) of normal epithelium, CIN and SCC, respectively. Co-expression of p16^{INK4a} and PCNA was seen in 85.7% (30/35) of samples. The combination of the three markers; laminin-5γ2 chain BM+ / p16^{INK4a} - / PCNA - was found in 5 of 8 (62.5%) samples with normal epithelium, laminin-5γ2 chain BM - / p16^{INK4a} + / PCNA + was found in 10 of 16 in CIN (62.5%). Only two samples with CIN had

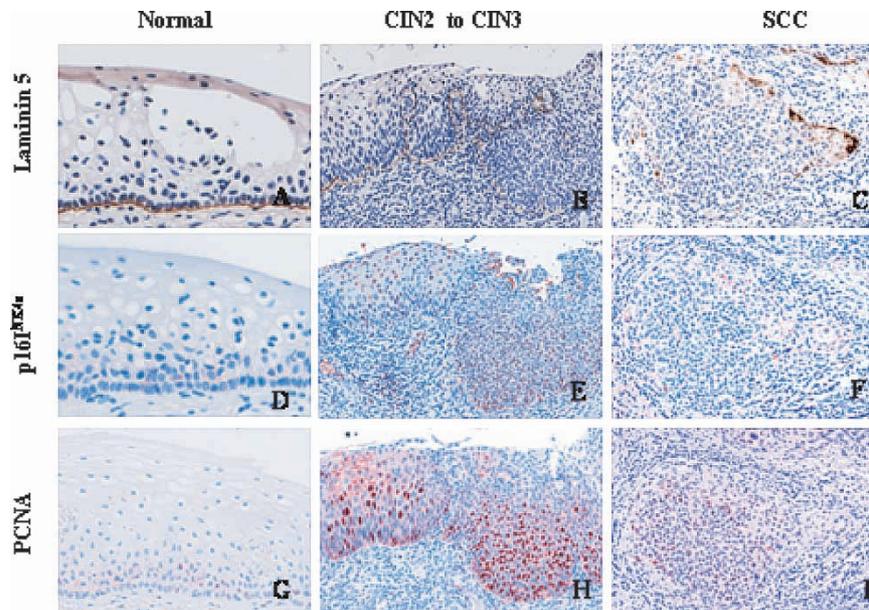


Figure 2. Expression of laminin-5γ2 chain, p16^{INK4a} and PCNA in samples from normal epithelium, different CIN stages, and invasive cervical cancer.

intracytoplasmic staining for laminin-5γ2 chain where p16^{INK4a} and PCNA expression were also positive simultaneously. Laminin-5γ2 chain Cyto+/p16^{INK4a} +/PCNA+ expression was seen in 63.6% (7/11) with SCC (results not shown). The co-expression of laminin-5γ2 chain, p16^{INK4a}, and PCNA during development of cervical neoplasia was showed in Table II. The cases that were

laminin-5γ2 chain BM -/p16^{INK4a} +/PCNA+ seemed to have the shortest interval time (average time 46.8 ± 36.3 months) for progression. However, the cases with combination of laminin-5γ2 chain BM+/p16^{INK4a} -/PCNA- have the longest time interval (average time 110.2 ± 52.7 months). A significant difference was seen between the two combinations (p < 0.05).

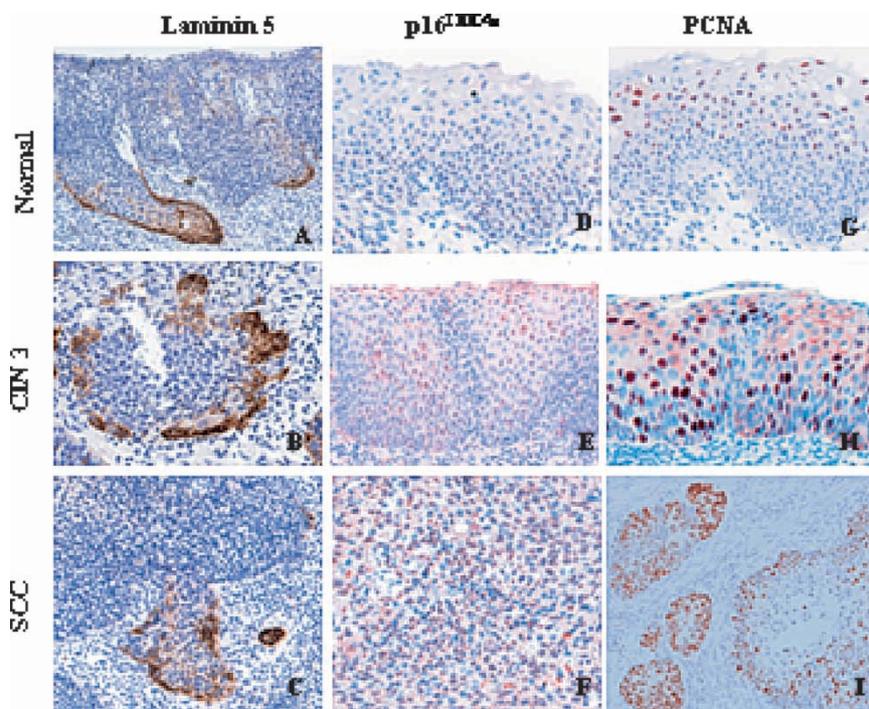


Figure 3. Coexpression of laminin-5 γ2 chain, p16^{INK4a} and PCNA in samples from normal epithelium, different CIN stages, and invasive cervical cancer.

Table II. Co-expression of Laminin 5, p16^{INK4a}, PCNA and the interval time for progression of cervical neoplasia

| Case | BM -/p16 ^{INK4a} +/PCNA + | | BM +/p16 ^{INK4a} -/PCNA - | | BM +/p16 ^{INK4a} +/PCNA - | | BM +/p16 ^{INK4a} -/PCNA + | |
|----------|------------------------------------|------------------------|------------------------------------|------------------------|------------------------------------|------------------------|------------------------------------|------------------------|
| | n | Interval time (months) |
| Normal 1 | 3 | 1 35 | 2 | 27*, 69 | 0 | | 0 | |
| Normal 2 | 5 | 0 | 3 | 126, 137, 173 | 2 | 58, 67 | 0 | |
| CIN+ | 7 | 3 16, 22, 55, 106# | 1 | 129 | 2 | 55 | 1 | 92 |

Normal 1: the disease progressed from normal squamous epithelium to cervical intraepithelial neoplasm (CIN).

Normal 2: from normal epithelium progressed to invasive cancer. CIN+: lesion of CIN progressed to invasive cancer.

*this case was diagnosed CIN2 76 months ago, then regressed to normal. After 27 months, patient progressed to CIN3.

#case 9: it was 104 months interval from CIN2 to CIN3, 2 months later, patient developed invasive cancer.

Discussion

The natural history of cervical cancer involves a number of events from the time of early detection of neoplasia to invasive cancer. The entire transformation process could take 13 to 20 years, affecting women in their mid forties or early fifties. HPV persistence has been established as a necessary cause for cervical cancer development [29] but HPV test alone has a low positive predictive value [30]. Several studies had shown that as the disease progressed from low grade CIN to high grade CIN or to invasive cancer, there was evidence of telomere attrition, an accumulation of genetic aberrations ultimately leading to aneuploidy, genomic amplifications, over expression of oncogenes, disruption of the cell cycle mechanism and imbalance of cell division [31–34].

The markers used in this study, laminin-5 γ 2 chain, p16^{INK4a} and PCNA were targeted to properties for tumor invasion, cell cycle disruption as a consequent of loss of p53 and/or pRB control, and as a result an increase in cellular proliferation. Kohlberger et al. reported basement membrane staining for laminin-5 γ 2 chain to be 69.4% to 77.8% in CIN1 to CIN3, and negative in normal epithelium and invasive cancer [16]. However, in our study, we found a reduction of basement membrane staining for laminin-5 γ 2 chain from 78.4% in normal epithelium to 41.2% in CIN1/CIN2, 27.8% in CIN3 and negative in SCC. Kohlberger et al. found no intracytoplasmic staining for laminin-5 γ 2 chain in normal epithelium of the cervix, while 2.8%, 20% and 30.9% of CIN1, CIN2 and CIN3 were laminin-5 γ 2 chain expression positive, respectively. We also found intracytoplasmic staining for laminin-5 γ 2 chain increased with cervical lesion progression [16]. This suggests that the loss of laminin-5 γ 2 chain in the basement membrane and the overexpression of laminin-5 γ 2 chain in the cytoplasm may be important steps in tumor cell invasion of cancer. Such changes in the expression pattern from being extracellular to intracellular of laminin-5 γ 2 chain and being more evident in cells located at the invasion front of high grade CIN and invasive

cancers further indicate that this marker is an excellent marker for progression to invasion. Alteration in laminin-5 γ 2 chain expression and its tissue distribution are able to reflect changes in epithelial-mesenchymal adhesion and integrity. Such alterations have been reported in oral squamous carcinoma, breast cancer, bladder carcinoma and invasive cervical cancer [18,35–37] which we also see in women with serial biopsies of lesions progressing from normal/low grade CIN to CIN3 and invasive cervical cancer. Furthermore, an overexpression of the laminin-5 γ 2 chain was associated with poor patient prognosis [38] and results from our previous study indicated that the γ 2 chain of the laminin-5 marker is more commonly expressed in squamous cell carcinoma of the cervix than in adenocarcinoma [39].

Previous studies have shown that p16^{INK4a} is strongly expressed in almost all cervical cancers. Nielsen et al. demonstrated p16^{INK4a} staining in some areas of squamous metaplasia with only relatively few Ki-67 positive cells in the same area [40]. Interestingly, we found co-expression of p16^{INK4a} and PCNA in the premalignant lesions and invasive cancer in our study. Moreover, the areas positive for p16^{INK4a} and PCNA overexpression were negative or only sporadically positive for laminin-5 γ 2 chain in the cervical cancer lesions of our material. Laminin-5 γ 2 chain positive cells were often located in the front of invasive cancer lesions, where p16^{INK4a} showed weak expression or was negative while PCNA had no expression at all within the same area. However Natarajan et al. reported 15 of 17 areas of dysplasia, microinvasion, and superficial margins of advanced oral squamous cell cancers had both p16^{INK4a} and laminin-5 γ 2 chain expressed together, and in 13 of these 15 lesions colocalization of laminin-5 γ 2 chain and p16^{INK4a} was obvious [41]. Cells at the epithelial-stromal interfaces of invasive SCCs lying deep in the dermal tissue typically expressed neither protein. The authors considered that p16^{INK4a} and laminin-5 γ 2 chain typically becomes expressed coordinately in

cells at locations just preceding and at the time of initial invasion into connective tissue. The difference between our data could be related to the different tissue type and the presence of HPV infection in our material.

Preinvasive lesions of the cervix are frequent, especially in young women, with a peak in incidence between the age of 25 and 40 years [42]. It was suggested that approximately 12.2% of all CIN3, when left untreated, would develop into invasive cancer with a mean duration of 13.3 years disease for the in-situ stage [43]. There is yet no reliable way to predict if a CIN lesion will progress to invasive cancer or if it will remain stable or regress to normal. Nordemar et al. noted that all six carcinoma-in-situ (CIS) lesions that progressed to invasive cancer within a follow-up time of five years, were laminin-5γ2 chain positive (100%), whereas only 10 of 27 lesions that did not progress, were positive (37%) ($p < 0.01$) in the larynx [44]. The authors suggest that a positive laminin-5γ2 chain laryngeal CIS lesion indicates a high risk for progression to invasive cancer. Skyldberg et al. also demonstrated that all microinvasive cancers and frankly invasive cancers had positive cytoplasmic staining for laminin-5γ2 chain, whereas only 11 of 32 lesions with CIN3 showed positive staining for laminin-5γ2 chain in the cells close to the basement membrane [18]. Most CIN1 and CIN2 lesions were negative for laminin-5γ2 chain at the basement membrane with exception to cases that progressed from high grade CIN to invasive cancer. In the present study, biopsies that were basement membrane negative for laminin-5γ2 chain but with overexpression of p16^{INK4a} and PCNA progressed to CIN or SCC require a shorter time for progression than those with positive basement membrane for laminin-5γ2 chain while the epithelium was negative for p16^{INK4a} and PCNA expression. In our previous study we reported the mean time taken for progression if only p16^{INK4a} was present, namely 64.2 months [24] while a combination of expression of these three markers used in this study had a time interval of gave a shorter mean time interval of 46.8 months.

In conclusion, in this study we could see that the loss of basement membrane and increased cytoplasmic staining for laminin-5γ2 chain is related to the severity of cervical neoplasia. Laminin-5γ2 chain strongly stained cells that might be involved with invasion of cancer cells. Cases with the combination of laminin-5γ2 chain BM⁻/p16^{INK4a}/PCNA⁺ have a shorter interval time for the progression of cervical neoplasia. Hence, the co-expression of laminin-5γ2 chain and p16^{INK4a} and PCNA simultaneously may be valuable for the prediction of cases that will progression.

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