E-cadherin, laminin and collagen IV expression in the evolution from dysplasia to oral squamous cell carcinoma

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ABSTRACT

Objectives: Study the loss or reduction of the cellular adhesion mediated for E-cadherin in oral leukoplakias, oral squamous cell carcinomas and metastatic nodules. Study the loss of continuity of the laminin and collagen IV expression in the epithelial basal membrane from the biological development of the oral leukoplakias and oral carcinomas.

Material and method: we have studied 124 samples of patient payees leukoplakias and oral carcinomas with diverse diagnosis that embrace from normal epithelium (13 samples), mild dysplasias (2), moderate dysplasias (12), "in situ" carcinomas (13), microinvasive carcinomas (11) oral squamous cell carcinomas (64 samples) and metastatic nodules (9). 7 blocks of tissue microarrays were built with needle of 2mm and was carried out a study by means of immunohistochemical technique for E-cadherin (clone 36, Biogenex), Laminin (078P, Biogenex) and Collagen IV (PHM12, Biogenex).

Results: In Mild and Moderate Dysplasias the results present loss of E-cadherin, Laminin, and Collagen IV (20%) expression. "in situ" and microinvasive carcinomas, the results presented loss of E-cadherin expression (73%), and loss in Laminin and Collagen IV expression (57%). In the squamous cell carcinomas , we find E-cadherin underexpression (90%) and discontinuity in the Basal Membrane. (70%). All the metastatic nodules presented loss of E-cadherin expression and discontinuity in Laminin and Collagen IV expression.

Conclusions: The loss of E-cadherin expression is increased when increasing the dysplasia grade of lesions. The loss of continuity in the laminin and Collagen IV expression follow a parallel evolution from dysplasias to metastatic nodules. The underexpression of the three markers has been significant in the evolution of the oral lesions.

Key words: *Leucoplasia, oral cancer, E-cadherin, Laminin, Collagen IV, tissue microarrays, immunohistochemistry.*

RESUMEN

Objetivos: Estudiar la pérdida o reducción de la adhesión celular mediada por E-cadherina en leucoplasias, carcinomas epidermoides y metástasis ganglionares. Estudiar la pérdida de continuidad de la expresión de laminina y colágeno IV en la membrana basal epitelial en el desarrollo biológico de las leucoplasias y carcinomas orales.

Material y metodo: Hemos estudiado 124 muestras de pacientes portadores de leucoplasias y carcinomas orales con diversos diagnósticos que abarcan desde epitelio normal (13 muestras), displasias leves (2), displasias moderadas (12), carcinomas in situ (13) carcinomas microinvasores (11) Carcinoma epidermoide oral (64 muestras) y metástasis ganglionar (9). Se construyeron 7 bloques de tissue microarrays con aguja de 2mm y se realizó un estudio mediante técnica inmunohistoquímica para E-cadherina (clona 36, T.D. ABD Company), Laminina (078P, Biogenex) y Colágeno IV (PHM12, Biogenex).

Resultados: En Displasias Leves y Moderadas presentan pérdida de expresión de E-cadherina, Laminina, y Colágeno IV (20%). En Carcinomas in situ y Microinvasores, presentaron pérdida de expresión de E-cadherina (73%), y en Laminina y Colágeno IV (57%). En los carcinomas epidermoides, encontramos pérdida de expresión de E-cadherina (90%) y discontinuidad en la M. basal (70%). Todas las metástasis ganglionares presentaron pérdida de E-cadherina y discontinuidad en Laminina y Colágeno IV.

Conclusiones: La pérdida de expresión de E-cadherina se incrementa al aumentar el grado de displasia de las lesiones. La perdida de continuidad en la expresión de laminina y Colágeno IV sigue una evolución paralela desde displasias a metástasis ganglionares. La disminución en la expresión de los tres marcadores ha sido significativa en la evolución de las lesiones orales.

Palabras clave: *Leucoplasia oral, cáncer oral, E-cadherina, Laminina, Colágeno IV, tissue microarrays, inmunohistoquímica.*

INTRODUCTION

Head and neck cancer represents around 3% of all neoplasias in United States, where it is estimated that more than 28260 new cases and 7230 annual deaths occurred in 2004 (1). Invasive nature and the ability to produce regional metastasis is an important prognostic factor and conditions the treatment of the patients with oral cancer (2). E-cadherin is a 120 kDa glycoprotein taking part in calcium-mediated cellular adhesion (3). Loss or reduction of E-cadherin mediated cellular adhesion is an important step in the development of invasion and metastasis in multiple carcinomas, included oral carcinomas (3-5).

The basal membrane is the first obstacle neoplasic cells should cross. The main components of the basal membrane are collagen IV, laminin and, in a smaller proportion, other molecules like perlecan, nidogen, entactin, collagen VII and others (6). In general the basal membrane is lost in many invasive carcinomas. The ability of malignant neoplasias to destroy the basal membrane has been correlated with its invasive potential and the loss of continuity of laminin expression and collagen IV can help us in early diagnosis and the prediction of the biological development of the oral lesions (7).

This study explored the behaviour of proteic expression of E-cadherin, Laminin, and Collagen IV in leucoplasias, squamous cells carcinomas of oral cavity and in metastasis to regional nodules in order to define a relationship among proteic expression of these markers with premalignant and malignant lesions of oral cavity. Tissue-micro arrays, multiple cores of samples for oral lesions in a same slide for inmunohistochemicals, were studied following this aim.

MATERIAL AND METHODS

For the realization of this study the files of the Pathologic and ENT (ear-nose-throat) Services of Clinical Universitary Hospital of Salamanca have been revised from 1990 to 2000. 124 samples were selected from patients with a diagnostic of oral leucoplasia informed by pathologists as 13 cases of normal epithelium, 2 mild displasias, 12 moderate displasias, 13 in situ carcinomas, 11 micro invasive carcinomas, 64 squamous cell carcinomas and 9 metastatic nodules following diagnostic criteria for these lesions (8,9). We recovered from files the slides of the selected biopsies and the paraffin conserved blocks of the 124 selected biopsies.

Construction of tissue-micro arrays:

We used Tissue-Arrayer™ device (Beecher instruments, Silver Spring, MD) (Fig 1A) It is a precision manually operated instrument that allows investigators to include multiple samples in a single block of paraffin. This way we can study multiple small samples under homogeneous conditions and with much smaller consumption of reagents than with conventional immunohistochemistry (10-12).

Study material was first selected among haematoxilin-eosine stained biopsy slides locating target areas (normal control tissue, hyperplasia, dysplasia, carcinomas, etc) (Figure 1B). Later on the previously selected area in the slides was identified in the donor paraffin block. A blank paraffin block was built to be used as a tissue-micro arrays receptor (fig 1C). We used two 2 mm section needles (donor and receptor). We placed receptor block inside its case and set zero position in X and Y micrometers (start point). A punch hole was made on receptor block with Tissue-Arrayer™ receptor needle and then we extracted the blank paraffin cylinder inside (fig 1D). Once the protector tray was placed over the receptor block,

the donor block conserved in paraffin was placed above it. We made a perforation in the preset area of the donor block with the donor needle of the Tissue-Arrayer, obtaining a core inside it, pressing the depth end above the punction needle. The protector tray was removed from the receiving block, and the core or was introduced in the hole that had been previously made in the receptor block (Figure 1E). The Tissue-Arrayer turret was progressed 3 mm following the "Y" axis and a new perforation was made with receptor needle repeating the steps above described, and so forth, until completing the first line of holes and occlusion with samples cores donors in the receiving block. A 3 mm lateral displacement was made following "X" axis from the last hole in the first row and then the first hole of the second row was performed. Then we repeated the steps described for the first line, in the second and successive lines (Figure 1F).

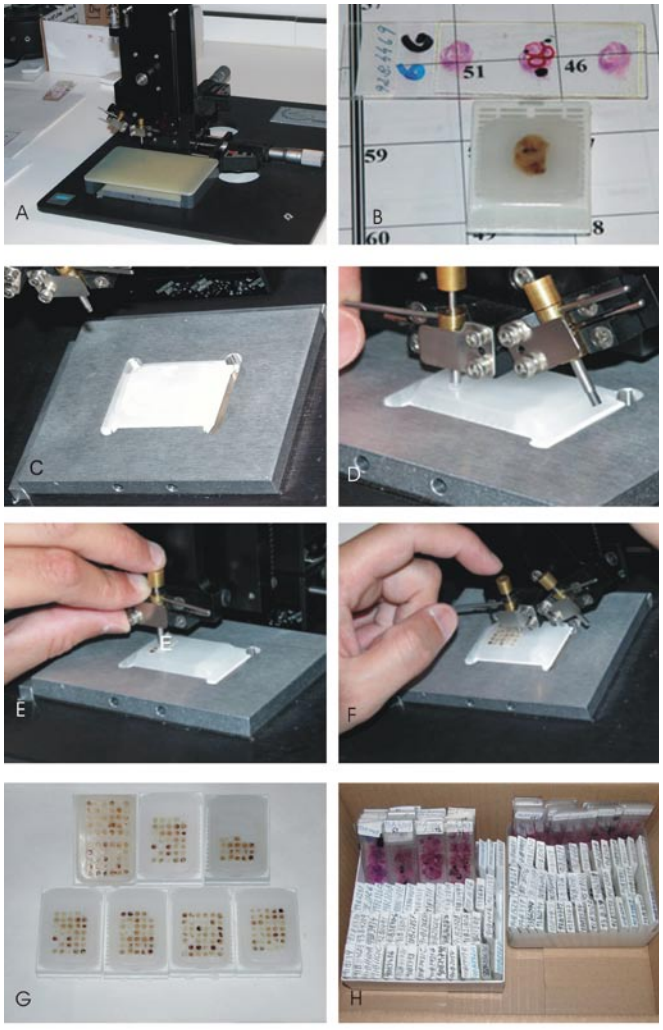


Fig. 1. Tissue Matrixes construction for the study of lesions in oral cavity

Once the first receptor Tissue-micro arrays block was completed we followed the same procedure till we obtained 60 cylindrical samples, 4 receptor blocks containing 35 cylinders (cores) each, a 42 cylinders block and one for the 18 cylinders left (Fig 1G). We included two cylindrical samples per patient (10), up to a total amount of 106 cylinders. This way we lay the whole of the duplicated samples on 3 blocks. Each one of these presents two blank orifices that will improve direction during microscopic examination.

Before making histological slices Tissue-micro arrays blocks were warmed up to 60 ºC for 10 minutes using a heater. Making this we smooth possible virtual spaces created in receptor blocks between receptor and donor orifices.

Inmunohystochemical techniques

Several 4 µm slices were obtained from the 7 micro-array blocks. Afterwards deparaffination and rehidratation of both, individual sample and multiple matrixes slides were performed using 5 minutes Xilol 100% baths up to four times followed by other three baths in absolute alcohol of 5 minutes each. Antigenic unmasking was performed by washing slides with non treated water before putting them into a pressure pot together with citrate buffer (pH 6) up to 3 minutes after boiling starts. Afterwards slides were washed using distilled water and then PBS.

Immunohistochemical marking technique was performed using an automatic device for inmunologic staining, *Optimax Plus* ® from *Biogenex, Menarini Diqagnostics*. Amplified Biothine/Estreptavidine technique (BSA) following supersensitive inmunodetection method from *Biogenex (San Ramon. USA)* was used. For E-cadherin we used clone 36 (1/200. B.D. Biosciences (San Diego, USA) as primary antibody; for laminin clone 078P (1/25, Biogenex); for collagen IV PHM12 clone (1/200, Biogenex). Incubation time was 30 minutes for both of them. The tissue was stained with Carazzi´s Hematoxylin and then assembled in a watery mean. In Negative controls primary specific antibodies were substituted by a buffer solution. A high laminin and collagen IV expression breast cancer tissue was used as Positive control.

Validation of immunohistochemistry

For E-cadherin we carried out a validation for two independent observers, as it follows: 3+ = complete stain of the cellular membrane for more than 50% of the coloured cells. 2+ = staining between 10% and 50% of cells. 1+ = less than 10% of coloured cells. 0 = stain absence (5). The pattern of laminin tint and collagen IV were evaluated to a 400X magnification along the basal membrane of normal mucose, dysplasic lesions and tumoral nests in carcinomas. Staining was classified as continuous when the reddish reaction of the product leaves along the epithelial and conjunctive border, or discontinuous when there was fragmentary or absent reaction. The areas of the epithelial disruption due to great inflammatory infiltrates of stroma were excluded from the study. Deposits of laminin and collagen IV around blood vessels were used as internal check (6).

STATISTICAL ANALYSIS:

Stat View 4.0 for Mac/OS statistical program was used. A descriptive statistical study (mean, typical deviation and standard error for quantitative variables) was performed. Inferential studies of central tendency comparison for quantitative variables were carried out using an ANOVA.

When it was significant ($p < 0,05$), the different parts were looked for using Bonferroni´s test, as well as Tukey´s and LSD. For qualitative or categorized variables the test we used was Chi-square. The determination of significance causes was made searching the maximum contributions to the contrast statistical and applying the Chi-square additive property. Contrast with $p < 0,05$ values were considered significant. Samples were classified in a normal epithelium group, a mild and moderate displasias group, a group for in situ carcinomas, a group for micro invasive carcinomas, a squamous cells carcinomas group and a metastatic nodules one.

RESULTS

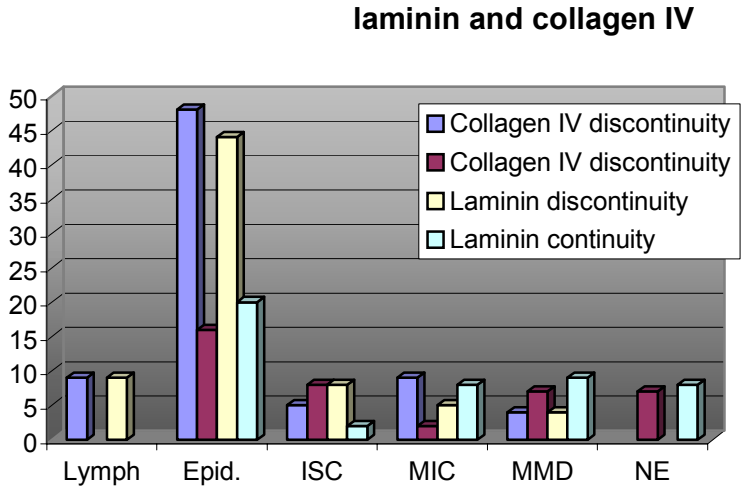
The ages of patients ranged from 34 to 94 years. 38,7% of the patients were individuals between 52 and 63 years old. 72,58% of the patients were male and 27,42% women.

Mild and moderate displasias presented E-cadherin, Laminin, and Collagen IV underexpression in 20% of the samples. In situ and micro invasive carcinomas, presented E-cadherin under-expression in 73% of the samples, and laminin and collagen IV under-expression in 57% of the samples. 90% of squamous cell carcinomas under-expressed E-cadherin and 70% exhibited discontinuity in the basal membrane. All metastatic nodules presented E-cadherin underexpression and discontinuity in laminin and collagen IV (Graphics 1 and 2).

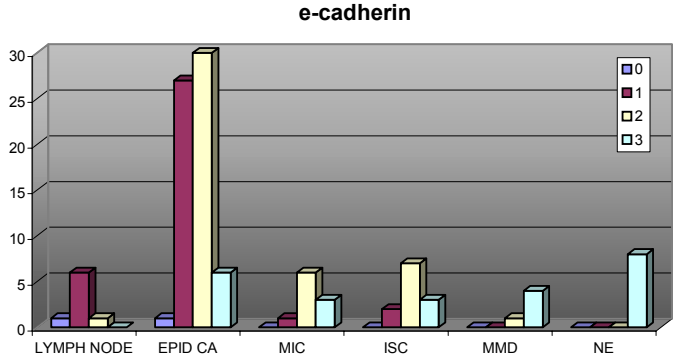
In light and moderate displasias we already observed an expression loss more evident in the group of carcinomas in situ and micro-invasive carcinomas. In the epidermoid carcinomas group an increase was observed in the lost of expression of E-cadherin that stayed the same in the ganglions group (Figure 2).

Significant differences in the expression levels of E-cadherin between normal epithelium group and in situ or micro-invasive carcinomas ($p < 0,002$) were found; The same occurred between normal epithelium and epidermoid carcinomas ($p < 0.001$). There were statistically significant differences between the group of light and moderate displasias and that of epidermoid carcinomas ($p < 0,001$) too so was between the group of in situ and micro invasive carcinomas and that of epidermoid carcinomas and metastatic nodules ($p < 0,01$).

In normal epithelium continuity of the basal membrane was observed (laminin and collagen IV). We began to observe discontinuity or occasional fragmentation in dysplasic lesions, in situ and micro invasive carcinomas. Scarce laminin and collagen IV expression surrounding the cords or nests of atypical epithelial cells was observed in squamous cell carcinomas (Figures 2). Laminin and collagen IV underexpression were total in the group of 9 metastatic nodules.



Graphic 1. Expression of Laminin and Collagen IV in normal Epithelium (EN), Mild and moderate Displasias (DLM), in situ carcinomas (CIS), microinvasive Carcinomas (CMI), oral squamous cell carcinomas and metastatic nodules.



Graphic 2. Expression of E-cadherin in oral lesions: normal Epithelium (EN), Mild and moderate displasias (DLM), "In situ" carcinomas (CIS), Microinvasive carcinomas (CMI), oral squamous cell carcinomas and metastatic nodules). An expression loss is observed that increases when advancing the displasic grade toward oral carcinoma and in metastatic nodules.

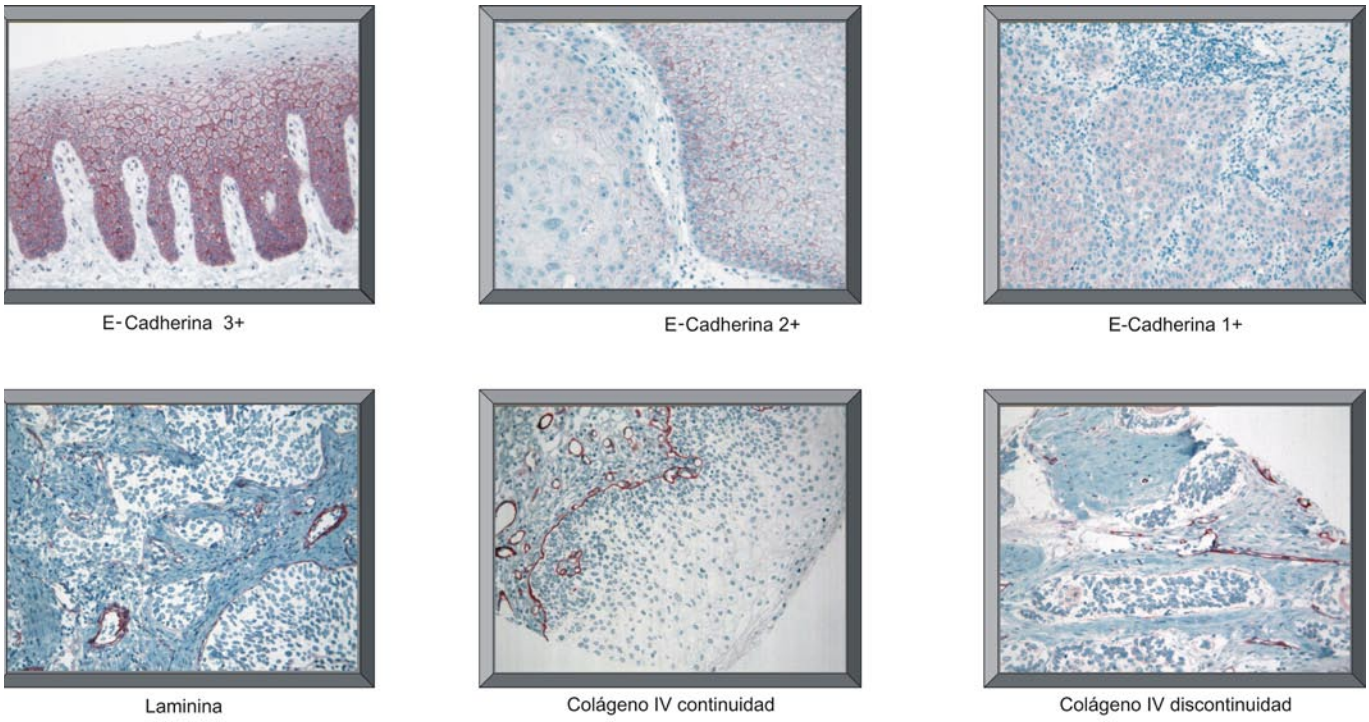


Fig. 2. E-cadherin, laminin and collagen IV staining patterns in oral lesions.

There were significant differences in the loss continuity of laminin and collagen IV observed between the squamous cell carcinomas group and precancerous lesions and between squamous cell carcinomas and the group including 9 metastatic nodules ($p<0,001$). Loss of continuity in the basal membrane studied in the groups of lesions by means of Laminin and collagen IV correlate significantly ($p<0,05$), when applying Pearson´s correlation coefficient.

DISCUSSION

E-cadherin plays an important role in the histogenesis and the maintenance of the structure of the normal epithelium. It forms "adherens junctions" (originally well-known as desmosomae belt) that form a continuous belt around the cell (4,13). The efforts to correlate E-cadherin with tumour development in oral carcinomas have not reached success. Most of carcinomas show variable grades of expression loss. However, this often is more frequent in poorly differentiated tumours. There are studies (2,14,15) that suggest that the E-cadherin underexpression is increased in invasion and metastasis in oral squamous cell carcinomas. Expression of E-cadherin it is neither correlated with the grade of tumour differentiation nor with metastasis. As in our case it has only been observed, expression reduction, as it always occurs in metastatic nodules so this is not a metastatic marker. Williams et al (16) found E-cadherin and/or catenin underexpression in the case of in situ carcinomas and infiltrative tumours.

They conclude that the rupture of the complex E-cadherin-catenin it is a late event associated with invasion. In summary the role of E-cadherin is to tie epithelial cells to each other and to maintain the integrity of the stratified epithelium. Generally, the most aggressive carcinomas show losses of epithelial cellular cohesion, and this is often associated with E-cadherin underexpression (4). In the Present study, E-cadherin underexpression was an early phenomenon as we observed in moderate displasias. This finding was more evident when "in situ" and micro invasive carcinomas are studied, showing that loss of epithelial cohesion can be an indicator of possible evolution. In squamous cells carcinomas and in metastatic nodules the expression levels of E-cadherin decrease considerably despite we didn´t found significant differences between both groups. This loss of expression increased as we advanced in the histopathological progression of the studied lesions.

Quantitatively, Laminin is the most important non collagenic proteic matrix in the basal membrane (7). Firth et al., showed that laminin and collagen IV distribution were continuous in epithelial hyperplasia while dysplasic lesions showed small focal breaks whose number increased in severe displasias (17). Kannan et al., reported a gradual increment in the frequency of laminin and collagen IV discontinuity from normal epithelium to hiperplasic, dysplasic and squamous cell carcinomas, with significant differences among groups (18). Harada et al. found that the stain pattern of laminin and collagen IV in primary oral squamous cell carcinomas was similar to that of metastatic nodules and observed that cellular population of the deep areas expressed the invasive and metastatic potential of oral carcinoma

(19). In our study we have found discontinuity in dysplasic lesions that progressively increased for "in situ" and micro invasive carcinomas being higher in oral squamous cell carcinomas and total in the group of metastatic nodules studied. On a practical approach infiltration should be the field of study of alterations of the basal membrane in oral squamous cell carcinomas. The results of the present study confirm that E-cadherin expression loss and laminin and collagen IV altered distribution in premalignant and malignant lesions of oral epithelium are synchronic with the progression of neoplasic transformation and thus are higher in metastatic nodules.

The technique of Tissue-arrays has shown to be an excellent technological medium for the study of multiple lesions of oral pathology. It allows visualization of multiple pathological lesions together with normal controls, saving of tissue and reactive markers allowing a bigger amount of studies with a smaller consumption. We must take in count that many of the studied samples come from incisional biopsies, where the quantity of available tissue is smaller than in pieces coming from surgical resections. Tissue micro-arrays are thus a promising way to carry out wide studies of multiple lesions and markers that lead us to a more precise knowledge of the alterations in the molecular biology of oral cancer.

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