Assessment of extracellular matrix proteins (laminin and fibronectin) in adenoid cystic carcinoma of salivary gland using morphometric method

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background

Adenoid cystic carcinoma (ACC) is an uncommon form of malignant epithelial neoplasm. It arises within major and minor salivary glands as well as mucous glands of the upper respiratory tract. It is characterized by high clinical malignancy, with unclear prognosis and unstable clinical course.

In histological examination of the tumour fibrillar structures are found in the extracellular matrix, containing fibronectin, type IV collagen and laminin.

Aim

The aim of the present study was to assess the protein components of the extracellular matrix – laminin and fibronectin.

Materials/Methods

The study group included 30 patients with ACC of major salivary glands. The expression of immunohistochemical reaction for laminin and fibronectin was assessed using computerized analysis of obtained images.

Results

The analysis showed no significant differences in mean laminin and fibronectin positive reaction areas. Analysis of the proteins depending on histological ACC subtype showed that fibronectin expression was significantly lower in tubular cancer compared to other types. For laminin no significant expression difference was found.

Conclusions

In the future the immunochemistry of laminin and fibronectin may become a useful element of prognosis assessment in patients with adenoid cystic carcinoma.

Key words

adenoid cystic carcinoma • laminin • fibronectin

BACKGROUND

Adenoid cystic carcinoma is an epithelial and highly malignant neoplasm. It develops from the epithelium of excretive parts of the salivary glands, although it may also arise in other primary sites. However, most commonly it develops in large and small salivary glands and mucous glands in the upper respiratory tract.

Its histological structure is rather complex. Microscopically, adenoid cystic carcinoma consists of small cells with large nuclei, which are located among the cystic and vesicular nests, giving it its specific appearance. These nests are filled with a PAS-positive substance arising from replication of basal lamina. It contains numerous fibronectin fibres, laminin, collagen type IV and other proteins of basal lamina. Also protein markers of cancer are produced there, e.g. carcinoembryonic antigen (CEA), S-100 protein and membrane protein (EMA) [1].

Fibronectin is a glycoprotein present in the human body in both soluble (in body fluids) and insoluble (forming fibres in the extracellular matrix, basal lamina, connective tissue and on the surface of various cells) forms. One very important feature of fibronectin is its ability to interact with other extracellular matrix components and to bind with macromolecules such as collagen and heparin as well as with human and bacterial cells. It is commonly believed that fibronectin plays an important role in adhesion of cells to the extracellular matrix and to other cells, thus mediating various cellular processes, e.g. proliferation, differentiation, migration and neoplasmatic transformation [2–5].

Laminin is the other protein characteristically found in the extracellular matrix of adenoid cystic carcinoma of salivary glands. It is also a glycoprotein, which generally plays a structural role, but its biological activity is very diverse. Laminin is one of the main components of basal lamina and it plays a major role in the process of interaction between epithelial and endothelial cells and the basal lamina. It is responsible for the growth, differentiation, morphology and migration of both normal and neoplasmatic cells [6–8].

AIM

The aim of the present study was to evaluate the extracellular matrix proteins of adenoid cystic carcinoma of the salivary glands using morphometric analysis.

MATERIAL AND METHODS

Tissue samples from 30 patients with adenoid cystic carcinoma were studied. The patients were diagnosed and surgically treated in the Otolaryngology and Oncological Laryngology Institute of the Medical University in Poznań. 21 subjects had a tumour of parotid, 8 of submandibular and 1 of sublingual salivary glands.

During immunohistochemical staining for laminin and fibronectin in light microscopy positive reaction was evaluated, with attention paid to distribution, alignment and intensity of staining, as well as to its relation to the parts of the tumour.

Quantitative assessment of immunohistochemical staining for laminin and fibronectin was performed using computer analysis of images. In order to assess the actual field stained for fibronectin and laminin photographical documentation was performed in the form of digital images with resolution of 640×480 pixels using light microscope with objective magnification 40× (Micro Optic Industrial Group Co), controlled using the software Motic Images v. 1.2 (MS Windows).

Fibronectin expression was measured with a method based on spatial representation of the positive staining reaction using A4D software developed in the Morphometry and Medical Images Processing Laboratory of the Medical University in Poznań. In this method a colour range (shades of brown) corresponding to a positive fibronectin reaction based on the brightness and saturation has to be selected. After the selection of positive reaction areas was performed, remaining image fragments were eliminated to the background level. The results expressed in pixels were subsequently represented as the percentage of positive reaction for fibronectin in the studied sample and also calculated into square micrometers (μm^2).

Morphometric analysis for laminin was performed using "Imane J" software v.1.32j. In the process 24-bit colour digital images with resolution of 640×480 pixels were transformed into 8-bit images with 256 shades of grey. On the latter images structures of interest were selected using so-called threshold segmentation. Due to the filamentous and branched nature of the studied glycoprotein, an additional skeletonization method was used in order to precisely delineate positive reaction areas on the processed images. On finally obtained binary (black-white) images
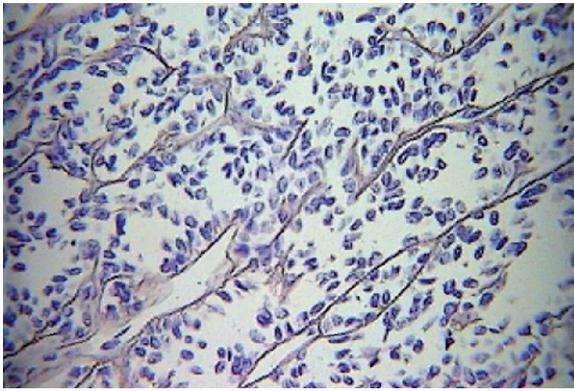
Figure 1. Immunohistochemical reaction for laminin in adenoid cystic carcinoma (ACC) of salivary gland. 24-bit colour image. Magnitude 400×.
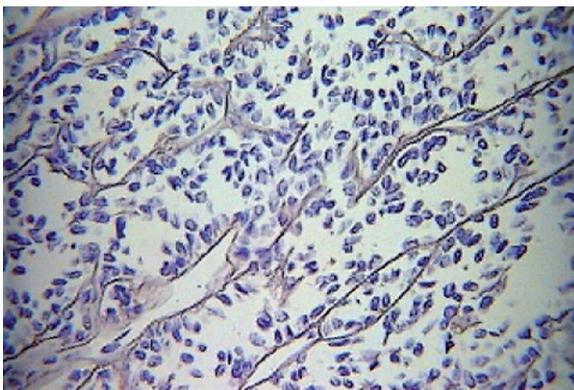

Figure 3. Threshold segmentation of immunochemical reaction for laminin. Binary image. Magnitude 400×.

Figure 2. The same image in greyscale (256 shades of grey). Magnitude 400×.
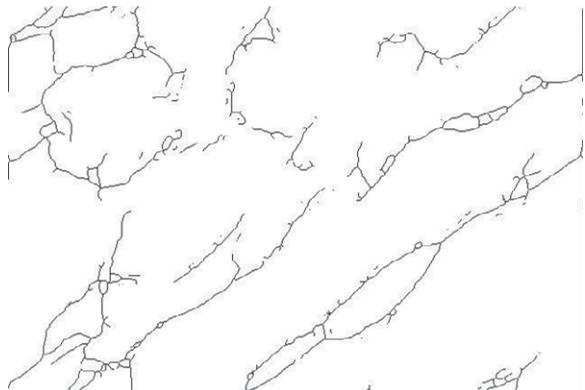
Figure 4. Laminin skeletonization. Binary image. Magnitude 400×.

the black areas corresponded to positive laminin reaction and white areas to background. The number of pixels of positive reaction areas was read out from the levels of grey histogram. Thus obtained numbers were calculated into percentage of positive reaction for laminin in the studied sample and also calculated into square micrometers (Figures 1–4).

RESULTS

In our series of samples the colour reaction for laminin was significantly weaker compared to fibronectin. In 7 cases the reaction was assessed as negative. In remaining 23 cases the delicate, filamentous structure of laminin was detected. The staining area was irregular, branching, fragmented and in most cases it did not surround the nests of cancerous cells. The image of reactions was similar in central and peripheral parts of studied samples.

The positive reactions for fibronectin were significantly stronger and were found in all 30 cases. The intensity of staining varied between the

samples, in most cases taking filamentous form. Fibronectin structures varied in length, taking irregular, chaotic forms. In some cases they were found inside the pseudocysts.

The area of positive reaction for fibronectin was on average 5205.3µm in the field of view (SD 2868.8µm). The area of positive reaction for laminin was on average 10467.8 (SD 28002.1µm). The difference in the areas of positive reactions for fibronectin and laminin was not statistically significant (p=0.34).

Evaluation of laminin and fibronectin expression in relation to histological subtype of adenoid cystic carcinoma was performed. Results are presented in Table 1.

In the cribiform subtype the area of positive reaction for fibronectin occupied 4.88% of the field of vision (with SD of 3.24%), in tubular subtype 2.46% (SD 1.28%) and in solid subtype 5.45% (SD 2.36%). Fibronectin expression in the tubular subtype was significantly lower than in cribiform (p<0.03) and solid subtypes (p<0.03). The

Table 1. Distribution of laminin and fibronectin in histopatological types of edenoid cystic carcinoma.

| Subtype	Average	Standard deviation (SD)
Fibronectin		
Cribiform	4.88	3.24
Tubular	2.46	1.28
Solid	5.45	2.36
Laminin		
Cribiform	1.58	1.32
Tubular	1.46	1.03
Solid	4.22	3.33

area of positive reaction for fibronectin did not differ significantly between cribiform and solid subtypes (p>0.6). There were no significant differences in laminin expression between histological subtypes; however, the strongest expression was observed for the solid subtype (in this subtype also the highest SD was found) (Figures 5,6).

DISCUSSION

Most authors believe that immunohistochemical staining for laminin and fibronectin may be useful in diagnosing adenoid cystic carcinoma. It may also have some prognostic value [8–14].

In our series laminin formed delicate, filamentous structures. The staining area was fragmented and in most cases it did not surround the nests of cancerous cells. The positive reaction for fibronectin was significantly stronger, taking filamentous form as well. Fibronectin structures took irregular, chaotic forms. They were often found inside the pseudocysts. Similar location was observed by most other authors [10,11,15,14].

D'Ardenne et al. analyzed 7 cases of adenoid cystic carcinoma of the salivary gland. In 6 cases they found large amounts of laminin and fibronectin dispersed irregularly between the neoplasmatic cells and inside the pseudocysts [10]. D'Ardenne suggested a close relation between laminin and fibronectin because of their "parallel microanatomical" alignment. In our series the areas taken up by these proteins did not differ significantly. In relation to histological subtypes, significantly weaker expression of fibronectin was observed in tubular cancer, compared to other forms. There are no data in the literature pertaining to the ex-
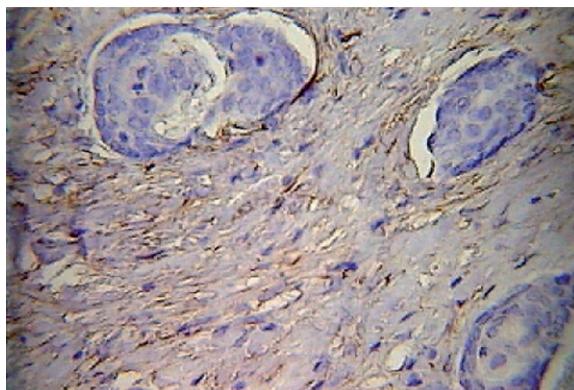
Figure 5. Immunohistochemical reaction for fibronectin in adenoid cystic carcinoma (ACC) of salivary gland. 24-bit colour image. Magnitude 400×.
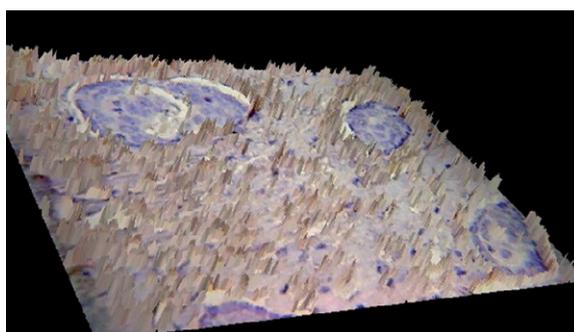
Figure 6. Segmentation of immunochemical reaction for fibronectin using spatial visualization technique. Magnitude 400×.

pression of discussed glycoproteins in relation to the histological cancer subtypes. Most authors believe that the tubular subtype is associated with better prognosis [16,13,17]. It is possible that laminin and fibronectin studies may open a new path for further immunohistochemical studies.

Dong examined the extracellular matrix of adenoid cystic carcinoma in 22 cases using immunohistochemical staining and electron microscopy. His observations were consistent with those of other authors – he found large amounts of both laminin and fibronectin in the matrix and inside pseudocyts.

Staining for components of basal lamina may be important for discrimination between invasive and non-invasive tumour [9–12,18]. Hua studied the role of laminin in invasiveness and metastasis of adenoid cystic carcinoma of the salivary gland. Two lines of cancer cells were evaluated: non-metastasing and highly metastasing, based on the assessment of receptors for laminin. Mobility of cells in Boyden's chamber was evaluated using the adhesion test. More laminin and laminin receptors were found in highly metastasing cell clones. Greater amounts of laminin also correlated

positively with migration ability. Low metastasing clones had stronger adhesion ability.

Other authors, who studied laminin expression in other types of cancer, confirmed these observations [6,12]. In studies on RCT sarcoma cells higher expression of receptors for laminin in highly metastasing cells was observed. Other cancers for which growth stimulation by laminin was found include: adenocarcinoma of the prostate gland, small cell lung cancer and Dunn's bone sarcoma [6].

In the assessment of malignancy of planoepithelial cancers of the head and neck (including cancer of salivary glands) their tendency for nerve invasion is also taken into consideration. The mechanism of nerve invasion is not fully understood, but the role of laminin is also suspected [8–10]. Preliminary studies suggest that removing elements of basal lamina is required for perineural infiltration [9]. Andersen studied 64 cases of planoepithelial cancers of the head and neck. He found a significant correlation between laminin expression and presence of perineural infiltration. This suggests that cancers with higher laminin expression may be more neurotropic.

CONCLUSIONS

Immunohistochemical assessment of laminin and fibronectin may give valuable information on prognosis in adenoid cystic carcinoma. The studies on the influence of these glycoproteins on cellular migration, and thus on local infiltration (especially of peripheral nerves) and systemic metastasis, are particularly interesting.

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