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Distribution of Basement Membrane in Supraglottic Carcinoma

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Abstract The object of this study was to assess the distribution of basement membrane in supraglottic squamous cell carcinomas. Expression of type IV collagen was detected by immunohistochemistry in resected supraglottic squamous cell carcinomas, and the correlation was examined between expression of type IV collagen and clinicopathological factors and cervical lymph node metastasis of supraglottic squamous cell carcinomas patients. An intact, continuous basement membrane was found in 17 cases (42.5%), while partial or widespread loss of the basement membrane was detected in the other 23 cases (57.5%). Heavily defective basement membrane was much more frequently observed in cases with poor histological differentiation ($P<0.05$). Cases with BM destruction were more likely to be accompanied by cervical lymph node metastasis ($P<0.05$). These data suggest that assessing the distribution pattern of basement membrane may be helpful in evaluating the malignancy grading of supraglottic squamous cell carcinomas and the potential occurrence of cervical lymph node metastasis.

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Keywords Supraglottic squamous cell carcinoma · Basement membrane · Type IV collagen · Cervical lymph node metastasis

Abbreviations

BM	Basement Membrane
SSCC	Supraglottic Squamous Cell Carcinomas

Introduction

Basement Membrane (BM) is a kind of material in extracelluar matrix that maintains the normal structure and function of organs. It has been observed by electron microscopy as a net-shape structure in a 3D irregular order, and it separates the layer of epidermis from the extracelluar matrix underneath [1]. Histologically, tumor invasion may be scaled as mild, moderate, severe dysplasia, and cancer in situ. The tumor cells penetrate the normal BM at the original spot, which is called invasion. After that, the tumor cells invade into adjacent tissue. Metastasis happens when the tumor cells enter the vessel system through BM around the vessel. Some studies have shown that tumor cells secrete enzymes to help penetrate the continuous normal barrier of BM [2-4]. Electron microscopic studies have proven that at the edge of invasive tumors, BM becomes thin, broken and shortened [5-7]. It has been suggested that BM is comprised of various components, including collagens, laminin, fibronectin etc. Among them, type IV collagen acts as the basic skeleton with all of the other components attaching onto it [8-10]. Recently, there have been several studies about the distribution pattern of BM in different organs but the conclusions are controversial.

In North-east China, as opposed to most other countries, supraglottic squamous cell carcinomas (SSCC) predominate

among the various types of laryngeal cancers. Supraglottic squamous cell carcinomas have a greater likelihood of lymphatic metastasis, which has a major impact on survival and have been noted as important prognostic indicators in laryngeal cancers. Here, we performed immunohistochemistry staining in supraglottic squamous cell carcinomas tissues using anti–human type IV collagen monoclonal antibody to assess BM integrity.

The data in this study show that heavily defective BM was much more frequently observed in cases with poor histological differentiation. Furthermore, the degree of BM loss correlated with cervical lymph node metastasis. These findings suggest that the distribution pattern of BM in SSCC may play an important role in tumor invasion and metastasis.

Methods

Material

Laryngeal squamous cell carcinoma tissues were taken from the tumor marginal area with adjacent tissues of 40 patients with supraglottic cancer, including twenty-four males and 16 females, aged from 50 to 79 years. Surgeries were performed at the 1st affiliated hospital of China medical university from 1999 to 2000. None of the patients had received pre-radiotherapy or chemotherapy. The pathological parameters were observed and the staging was based on the TNM classification of UICC in 2002. All tissues were taken through ipsilateral or bilateral neck dissection, and lymph nodes isolated to determine whether lymph node metastasis had occurred.

Five normal laryngeal mucosa tissues used as control were taken from normal part of total laryngectomy sample.

The study protocol was approved by the local Ethics Committee.

Immunohistochemistry

Tissues were fixed in 10% formaldehyde solution and embedded in paraffin. Each sample was cut into 5 μm continuous slices and deparaffinized with xylene, then the sections were rehydrated through serial dilutions of ethanol to distilled water. To improve antigen exposure, sections were enzymatically pretreated with 0.4% pepsin (Sigma) for enhancement of immunostaining [11]. Sections were incubated in blocking buffer [5% bovine serum albumin in phosphate-buffered saline (PBS)] for 1 h followed by primary antibody diluted in blocking buffer at 4°C overnight (monoclonal mouse antibody against type IV collagen, Maxim Biotech Inc, Beijing, CN; 1:100). After three washes in PBS, a streptavidin-biotin complexed specific secondary antibody (Maxim Biotech Inc) was applied to the sections for the visualization of antigen as described. Finally, sections were lightly counterstained with hematoxylin to facilitate identification of cell nuclei. Photomicrographs were taken using a microscope-mounted Nikon FX-35WA camera (Nikon Co., Tokyo, Jp) and Kodak Ektachrome film (Eastman Kodak Co., Rochester, NY, U.S.A.). The digital images were prepared for publication using Adobe Photoshop 7.0 software (Adobe Systems, Inc., San Jose, CA, U.S.A.). For negative controls, sections were similarly processed but without primary antibodies, and demonstrated negligible levels of non-specific background.

Analyses of all slices were performed under the supervision of two pathology physicians. For statistical analysis, 10 area units per slice showing typical characteristics of squamous cell carcinoma were carefully selected, and three slices were used for every case. The ratio of the number of continuous type IV collagen-surrounding cancer cell nests to the total number of cancer cell nests was then calculated for each unit, and X^2-test was used.

Results

Type IV collagen has a brown linear structure, as observed in the positive control tissues. BM integrity was determined based on the distribution pattern of the type IV collagen in the tissue of SSCC, as shown in Fig. 1. Intact BM (++, continuous brown linear structure as shown in Fi.g 1a); partial BM (+, missing over 50% discontinuous brown linear structure as shown in Fig. 1b); widely missing BM (-, only part brown linear structure was left or can not be recognized as shown in Fig. 1c).

In the normal control tissues, as shown in Fig. 1d, the distribution of continuous brown linear structures could be seen between the epithelium and the underlying matrix. The distribution of continuous linear structures could also be seen around the small blood vessels and small lymphatic vessels. However, no color was seen within the vessels or in the nuclei of cells. The expression patterns of type IV collagen were similar in tumor tissues. However, among all SSCC cases, there was a notable difference between the strength of distribution and continuity. Samples in this study included 17 (++) cases (42.5%), 14 (+) cases (35%), and 9(-) cases (22.5%).

In highly differentiated tumors, the tissues showed a relatively continuous expression of BM, as shown in Table 1. Half of the samples showed intact BM expression, and only 6.3% showed widely missing BM. The percentage of poorly-differentiated tumors associated with BM depletion (12.5%+62.5%) was notably higher than that observed with moderately–or highly-differentiated tumors (37.5%+18.8%, and 43.7%+6.3%, respectively). This difference is

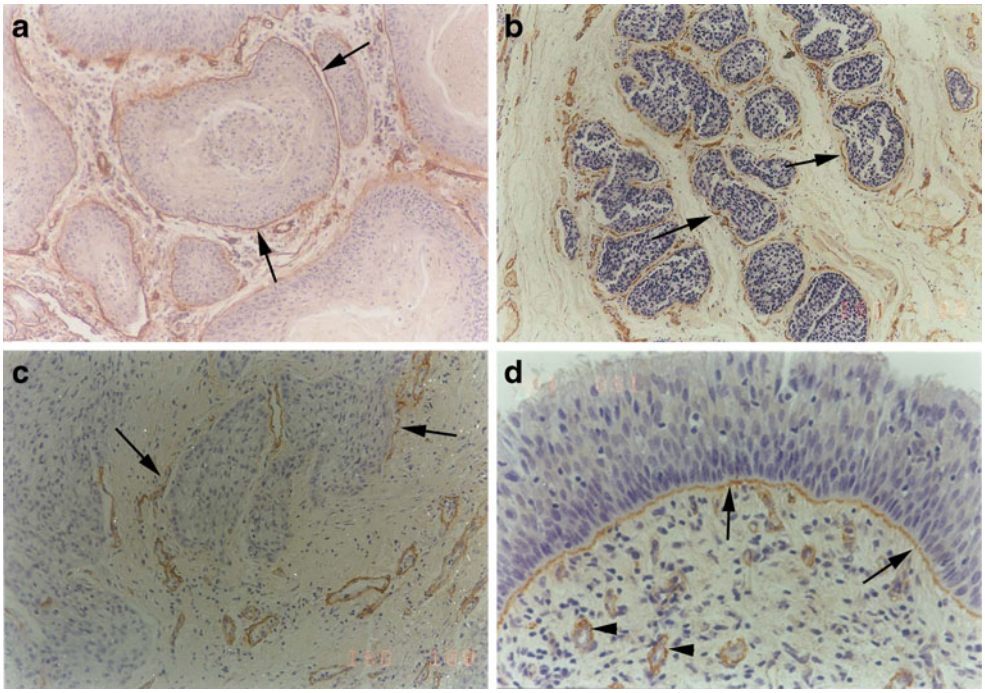

Fig. 1 Distribution pattern of the type IV collagen in the tissue of SSCC and normal control. As shown in a: continuous brown linear structure was seen around tumor cells (*arrow*); in b: discontinuous brown linear structure was detected (*arrow*); in c: only part brown linear structure was left (*arrow*); in d: the distribution of continuous brown linear structures could be seen between the epithelium and the underlying matrix in normal tissue (*arrow*) also be seen around the small blood vessels and small lymphatic vessels (*arrow head*)

even more apparent upon comparison of samples where the BM is completely absent. Statistical analysis showed that this difference is significant ($P<0.05$).

To evaluate the distribution of BM in different tumor invasion patterns, we used the Yamamoto grading system [12] to assess tumor invasion (see Table 2). Our results show that in parallel with increasing degrees of dispersion and tumor invasion, the detection of intact BM decreased. Most G1 (see Table 2) tumors were surrounded by a completed edge (83.3%), while only a few tumors maintained an intact BM in G4C (27.3%). The percentage of tumors with a relatively continuous edge (G1G2) that maintain an intact BM is 64.2%, while for tumors with a dispersed edge (G3G4), only 30.8% maintain an intact BM (shown in Table 3). The difference was significant ($P<0.05$). The results above demonstrate the strong relationship between the integrity of BM and the degree of tumor invasion.

Among the cases with lymph node metastasis, only a few cases were surrounded by a complete BM (22.2%); rather, most of them exhibited partial or widely absent BM (as shown in Table 4). However, in the cases without lymph node metastasis, nearly 60% showed an intact BM pattern, and very few tumors completely lost BM (4.5%). Among the patients with a normal distribution of BM, only 23.5% experienced lymph node metastasis, while among the cases where BM was lost completely, the rate is 88.9%. Statistical analysis showed the difference to be significant ($P<0.05$).

We also found that with the development of clinical stage from I to IV, the (++) distribution of BM decreased gradually while the depletion and shortening of BM (+) and (-) increased. However, the difference was not significant ($P>0.05$) based on the X^2-test (Data not shown).

Table 1 Distribution of basement membrane in tumor with different differentiated degree

BM	Tumor differentiated degree		
	Highly	Moderately	Poorly
++	8(50.0%)	7(43.7%)	2(2.5%)
+	7(43.7%)	6(37.5%)	1(12.5%)
–	1(6.3%)	3(18.8%)	5(62.5%)

$x^2 = 9.996$, $P<0.05$

Table 2 Tumor invasion mode (Yamamoto 1983)

Grade	Criteria
G1	Well defined borderline
G2	Cords, less marked borderline
G3	Groups of cells, no distinct borderline
G4	Diffuss invasion
	Cord-like type (4C)
	Widespread type (4D)

Table 3 Distribution of basement membrane in different tumor invasion pattern

BM	Tumor invasion mode				
	G1	G2	G3	G4C	G4D
++	5(83.3%)	4(50.0%)	5(83.3%)	3(27.3%)	0(0%)
+	1(16.7%)	4(50.0%)	6(46.1%)	3(27.3%)	0(0%)
–	0(0%)	0(0%)	2(15.4%)	5(45.4%)	2(100%)

X^2=4.18, P<0.05

Discussion

BM is the extracelluar matrix outside the cells, located under epidermal cells and endothelial cells. It is a combination of the secretion of epidermal, endothelial, and smooth muscle cells and is found in different kinds of organs. Under normal conditions, BM is a stable, compact, continuous structure with selective filtering quality. Only material with small molecules are able to penetrate. Cells and material with bigger molecules can only penetrate BM when some special pathological circumstances occur, such as inflammation and cancer, when the BM is partially damaged or missing. Research has shown that BM affects the conformation and the biochemical function of cells through mechanical and chemical means [10, 13-15]. The cross-effect between the normal cells and BM changes during tumor development, and thus affects the proliferation and invasive capacity of tumors. There has been much attention paid to the relationship between tumor biology behavior and BM. Some researchers have suggested that tumor invasion might be divided into three steps: first, the special receptor on the surface of the tumor cells attach onto BM by combining with special proteins in the middle layer of BM. Then the tumor cells secrete collagen enzymes to decompose the BM. Finally, the tumor cells penetrate BM, accessing nearby tissue [4]. Research on galactophore, head and neck cancer show that the tissue on either sides of the BM do not affect each other in situations of typical dysplasia and in situ cancer. In tissues exhibiting micro-invasion, BM is observed to be partially damaged or missing [16-19].

Our results have shown that about 57.5% of the population have partial loss or widespread loss of BM, while about 42.5% population have intact BM, which proves that the loss of BM does not occur in all invasive SSCC. Our study also shows that the cancer nest is consistently surrounded by intact BM in the tissue of highly-differentiated tumors, while BM loss is more significant in proximity to the tissue of poorly-differentiated tumors. This result suggests that the squamous cancer cells maintain the same ability as healthy epidermal cells to produce BM compound. The depletion of BM may be caused by the following reasons: 1.the decreased ability of tumor cells to produce BM compound; 2. over-production by tumor cells of the enzyme used to decompose BM; or 3. the ambient inflammatory cells invade and secrete digestive enzymes which can destroy BM. Research has shown that when BM depletion occurs, the response of the ambient inflammatory cells is enhanced, which is consistent with the third hypothesis [4]. Other studies have shown that the tumor cell-secreted enzyme of type IV collagen, which is positively correlated with the destruction of BM, is obviously increased [1-4]. And an electron microscopic study showed that a progressive fibrillogenesis with an activation of the connective tissue elements of the border was formed in specimens with supraglottic carcinomas [20]. Thus, depletion of BM may result from the cooperation of multiple—factors, and still needs to be studied in the future. Although we do not address the cause of the depletion of BM in this study, our results prove that BM tends to be lost as the differentiation of SSCC worsens. Also, the fact that the inflammatory cells can secrete enzymes to digest BM should be considered.

The main characteristics of malignancy are invasion and metastasis. The pattern of tumor invasion is closely related to metastasis and prognosis. In this study, by investigating the relationship between the BM distribution pattern and the invasion pattern in SSCC, we found that in the tissue with G1 invasive patterns of SSCC, the BM distribution pattern is nearly intact, while in the tissue that does not have an obvious invasive edge, but rather exhibits a dispersed invasion pattern of the tumor cells (G4), less BM is observed in the extracelluar matrix around the tumor. These results show that the loss and shortening of BM is serious in the cases that have stronger invasive ability; in other words, tumor tissue with more serious BM deficiencies has more of a chance to invade the adjacent tissue. The strong consistency observed in this study suggests that the distribution pattern of BM can be used as a criterion to assess tumor invasive ability in SSCC.

Our previous results have shown that SSCC with a high degree diffuse invasion have a significantly higher proportion of regional lymph node metastasis than less invasive tumors [21]. As observed in our study, tumor cells invade into the ambient matrix, then enter the vascular system by breaking down the BM that surrounds the small vessels, so

Table 4 Distribution of basement membrane in tumor with lymph node metastasis

BM	Lymph node metastasis	
++	4(22.2%)	13(59.1%)
+	6(33.3%)	8(36.4%)
–	8(44.5%)	1(4.5%)

X^2=10.197, P<0.05

the metastasis occurs. Meanwhile, the close relationship between the integrity of the BM that surrounds the cancer and the lymph node metastasis in the neck was also demonstrated. Our results support the hypothesis: cancer surrounded by intact BM has a much lower potential for metastasis, while in the absence of an intact BM, the tumor cells can penetrate the BM barrier and metastasis occurs. The results of the current study suggest that the distribution pattern of BM can be used as one of the important parameters to predict lymph node metastasis in SSCC.

Tumor biology behavior is important for the prognosis of the patient. Both the cell differentiation and the degree of invasion should be used as important histological parameters to assess cancer behavior. The current study shows that the two standards of the degree of malignant tumor tissue: the degree of differentiation of the tumor cell and the pattern of invasion, both have a strong relationship to the BM distribution pattern. And the distribution pattern of BM can be used as one of the important parameters to predict lymph node metastasis in SSCC. Considering that neck lymph node metastasis is the most essential factor that affects future prognosis for head and neck cancer, the measurement of the BM distribution pattern in cancer is then not only the evidence of invasion but also the representation of the internal behavior of cancer and can be used to assess the biological characteristics and degree of malignancy in some level.

Meanwhile, we studied the relationship between the clinical stage and the BM distribution pattern and found that the degree of depletion and shortening of BM tends to relate to the clinical stage but the relationship is not statistically significant. Future research needs to be done.

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References

1. Brown PD, Levy AT, Margulies IM, Liotta LA, Stetler-Stevenson WG (1990) Independent distribution pattern and celluler processing of the 72 KD type IV collagenase and interstital collagenase in human tumorgenic cell lines. Cancer Res 50:6184–6191
2. Hasty KA, Pourmotabbed TF, Goldberg GI, Thompson JP, Spinella DG, Stevens RM, Mainardi CL (1990) Human neutrophil collagenase. A distinct gene product with homology to other matrix metalloproteinases. J Biol chem 265:11421–11424
3. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, Kronberger A, He CS, Bauer EA, Goldberg GI (1988) H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem 263:6579–6587
4. Lunt SJ, Chaudary N, Hill RP (2009) The tumor microenvironment and metastatic disease. Clin Exp Metastasis 26:19–34
5. Hay ED (1982) Cell biology of ertracellular matrix. Plenum, New York
6. Liotta LA (1984) Tumor invasion and metastases: role of the basement membrane. Am J Pathol 117:339–348
7. Leblond CP, Inoue S (1989) Structure, composition and assembly of basement membrane. Am J Anat 185:367–390
8. Sakr WA, Zarbo RJ, Jacobs JR, Crissman JD (1987) Distribution of basement membrane in squamous cell carcinoma of the head and neck. Hum pathol 18:1043–1050
9. Carter RL, Burman JF, Barr L, Gusterson BA (1985) Immunohistochemical localization of basement membrane type IV Collagen in invasive and metastatic squamous carcinomas of the head and neck. J Pathol 147:159–164
10. Martinez-Hernandez A, Amenta PS (1983) The basement membrane in pathology. Lab Invest 48:656–677
11. Barsky SH, Siegal GP, Jannotta F, Liotta LA (1983) Loss of basement membrane components by invasive tumors but not by their begin counterparts. Lab Invest 49:140–147
12. Yamamoto E, Kohama G, Sunakawa H, Iwai M, Hiratsuka H (1983) Mode of invasion, bleomycin sensitivity and clinical course in squamous cell carcinoma of the oral cavity. Cancer 51:2175–2180
13. Radisky DC (2005) Epithelial-mesenchymal transition. J Cell Sci 118:4325–4326
14. Bienz M (2005) beta-Catenin a pivot between cell adhesion and Wnt signalling. Curr Biol 15:64–67
15. Bonitsis N, Batistatou A, Karantima S, Charalabopoulos K (2006) The role of cadherin/catenin complex in malignant melanoma. Exp Oncol 28:187–193
16. Andreasen PA, Egelund R, Petersen HH (2000) The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci 57:25–40
17. Osinsky SP, Ganusevich II, Bubnovskaya LN, Valkovskaya NV, Kovelskaya AV, Sergienko TK, Zimina SV (2005) Hypoxia level and matrix metalloproteinases-2 and–9 activity in Lewis lung carcinoma: correlation with metastasis. Exp Oncol 27:202–205
18. Rofstad EK, Rasmussen H, Galappathi K, Mathiesen B, Nilsen K, Graff BA (2002) Hypoxia promotes lymph node metastasis in human melanoma xenografts by up-regulating the urokinase-type plasminogen activator receptor. Cancer Res 62:1847–1853
19. Visser R, van der Beek JM, Havenith MG, Cleutjens JP, Bosman FT (1986) Immunocytochemical detection of basement membrane antigens in the histopathological evaluation of laryngeul dysplasia and neoplasia. Histopathology 10:171–180
20. Répássy G, Czigner J, Ribári O, Lapis K (1988) The barrier-like role of activated connective tissue against the spread of supraglottic carcinoma. Arch Otorhinolaryngol 245:151–154
21. Wang Y, Tian J, Ren Z (1997) The relationship between histologic grading of malignancy and regional lymph node metastasis in squamous cell carcinoma of larynx. J Clin Otorhinolaryngol (China) 11:160–162