Original article
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# The prognostic significance of type IV collagen expression in colorectal carcinomas

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**BACKGROUND:** *Breakdown of basement membrane is believed to be an essential step for tumor invasion and metastasis. The interaction between tumor cells and extracellular matrix can also result in induction of basement membrane synthesis by tumor and stromal cells. The aim of this study was to investigate distribution patterns of type IV collagen expression at the basement membrane in the tumor tissue of colorectal carcinomas by immunohistochemical staining. The degree of expression of type IV collagen correlated with classical clinicopathologic prognostic factors and their potential relationship with patients' prognosis.*

**PATIENTS AND METHODS:** *This study included 40 patients who underwent curative resection of colorectal cancer at the Department of Surgery in the General Hospital Senta with complete follow-up for 5 years or until death. The identification of basement membrane was performed immunohistochemically using monoclonal antibodies to collagen IV. The basement membrane synthesis at the invasive front of colorectal cancer was semiquantitatively assessed as mild, moderate or severe.*

**RESULTS:** *The deposition of basement membrane type of collagen IV had a statistically significant correlation with the stage of disease and histological grade of tumor with tendency of lesser synthesis of basement membrane in the advanced stage of disease and poorer histological grade of tumor. There were no significant correlations between intratumoral basement membrane synthesis and sex and age of patients, and localization and histological type of tumor. Five-year survival rates in patients with mild synthesis of basement membrane in colorectal tumor were significantly lower than in patients with severe expression of basement membrane. The basement membrane synthesis in colorectal cancer was a prognostic factor with significance behind stage of disease and histological grade of tumor by univariate Cox hazard-model. Multivariate Cox analysis verified that explicit synthesis of basement membrane showed a trend toward better prognosis, but it was not statistically significant.*

**CONCLUSION:** *Immunohistochemical staining for type IV collagen is an important additional technique with prognostic value.*

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**KEY WORDS:** *Colorectal Neoplasms; Collagen; Basement membrane; Prognosis*

## INTRODUCTION

The colorectal carcinoma (CRC) is a leading malignant tumor of the gastrointestinal tract and second major cause of cancer-associated morbidity and mortality (1). The prognosis of CRC is, in general, predicted by the well-established Dukes' classification based on the two most powerful prognostic indicators: penetration of the bowel wall and local lymph node involvement (2). The Dukes' staging system remains the most important determinant of the decision to institute postoperative chemotherapy in both colonic and rectal cancer (3). Yet, in each Dukes' class, the survival of individual patients may vary considerably. This may occur because the classification reflects a stage in course of CRC rather than the biological behavior of the neoplasm (4). The differences in the survival rate of patients with the same stage of CRC disease have induced a search for new diagnostic

Abbreviations:

CRC - colorectal carcinoma; ECM - extracellular matrix; BM - basement membrane

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methods with prognostic relevance.

Interactions of tumor cells and extracellular matrix (ECM) components are crucial determinants of tumor cell spreading and metastatic activity. Breakdown of basement membrane (BM) is believed to be an essential step for tumor invasion and metastasis (5). Transition from in situ to invasive carcinoma implies BM breakdown and passage of tumor cells through the BM. BM forms the boundary between the epithelial compartment of tumors and adjacent stroma. In the process of invading and metastasizing carcinomas must penetrate or lose their epithelial BM, and penetrate BM of the surrounding lymphatic and blood vessels. The situation in somewhat complex, however, as interaction between tumor cells and ECM can also result in induction of matrix synthesis by tumor and stromal cells (6).

Various disorders of BM patterns have been described in human carcinomas. Collagens and laminin are major components of BM involved in cell-matrix interaction and tumor progression. Laminin and collagen have been studied in several tumor types and their immunomorphological expression correlated with tumor morphogenesis, local invasiveness, and metastatic behavior (7-10). The disruption and absence of BM may reflect abnormal synthesis of BM components or abnormal lysis due to proteases produced by tumor cells (5,11). In addition, collagen type IV immunostaining facilitates recognition of vascular invasion by highlighting the BM of vessels (4,9).

The aim of this study was to investigate distribution patterns of type IV collagen expression at the basement membrane in the tumor tissue of colorectal carcinomas by immunohistochemical staining. The degree of expression of type IV collagen correlated with classic clinicopathological prognostic factors and their potential relationship with patients' prognosis.

## PATIENTS AND METHODS

The study population included 40 patients who underwent curative resection of CRC at the Department of Surgery of General Hospital Senta from 1990 to 1995. None of them had received chemotherapy or radiation therapy before surgery. The operations were standard colon or rectum resections with regional lymph node dissection.

The tumors were typed and graded according to the criteria of the World Health Organization classification (12). The extent of the tumor invasion and metastasis were based on the Astler-Coller modification of the Dukes classification system (13). Patients who died within 30 days after surgery were not included in the study. Only patients classified as stage C received postoperative chemotherapy using 5-fluorouracil and leucovorin.

Slides were obtained from the invasive front of tumor (14). Sections thick 3 $\mu$m were cut from the selected original paraffin blocks, and rehydrated in usual manner. Immunohistochemical staining was carried out in the Ventana ES automated immunohistochemistry system (Ventana Medical System Inc., Tucson, AZ, USA) using original Ventana reagents, with the exception of the primary antibodies, Collagen IV (DAKO M 0785, DAKO, Glostrup, Denmark). Antigen retrieval was performed with 20 minutes protease 1 digestion. Collagen IV was used at the dilution of 1:300 with reaction time of 32 minutes. The slides were weakly counterstained with hematoxylin and were routinely mounted. Using antibodies to type IV collagen, synthesis of BM around tumor nests at the tumor-stromal border was immunohistochemically visualized and semiquantitatively assessed. The amount of type IV collagen expression was scored as mild (score 1 - synthesis of complete BM around less of 25% of tumor nests), moderate (score 2 - synthesis of complete BM around 25% to 75% of tumor nests), and severe (score 3 - synthesis of complete BM around more than 75% of tumor nests), as illustrated in Figure 1. All patients were followed up every third month for 3 years after surgery and subsequently every sixth month for at least 5 years, or until death.

Statistical analyses were performed using computer system for biomedical investigation MedCalc (MedCalc Software, Mariakerke, Belgium). A value of $p<0.05$ was considered statistically significant. Student's *t* test was used to evaluate differences of the mean age. Collagen IV expression was compared to various clinical and histopathologic parameters by menas of nonparametric Kruskal-Wallis test. Survival curves were calculated using Kaplan-Meier method and analyzed by log-rank test. The influence of each variable on survival was assessed by the Cox proportional hazard model. Univariate Cox regression analysis was performed to determine if the prognostic variables were predictive of overall survival. Stepwise Cox regression was used to determine which combination of prognostic variables was most suitable as independent predictors of overall survival. Univariate and multivariate Cox analysis was made using BMDP (BMDP Statistical Software Inc., Los Angeles, CA, USA) program package.

## RESULTS

The study was based on 40 patients (24 men, 16 women) with CRC. The age of the patients ranged from 33 to 82 years, with mean age of $62.7\pm8.94$ years. BM was identified by reddish brown coloration with collagen type IV antibody. The strong and well-defined type IV collagen staining was seen in the BM of normal glandular epithelium and around blood vessel walls. The epithelial cells showed negative reaction. In tumor specimens BMs were frequently irregular, discontinuous and crumbled. There was an increased expression of collagen IV in fibrillar components of tumor stroma. Mild expression of BM in the colorectal

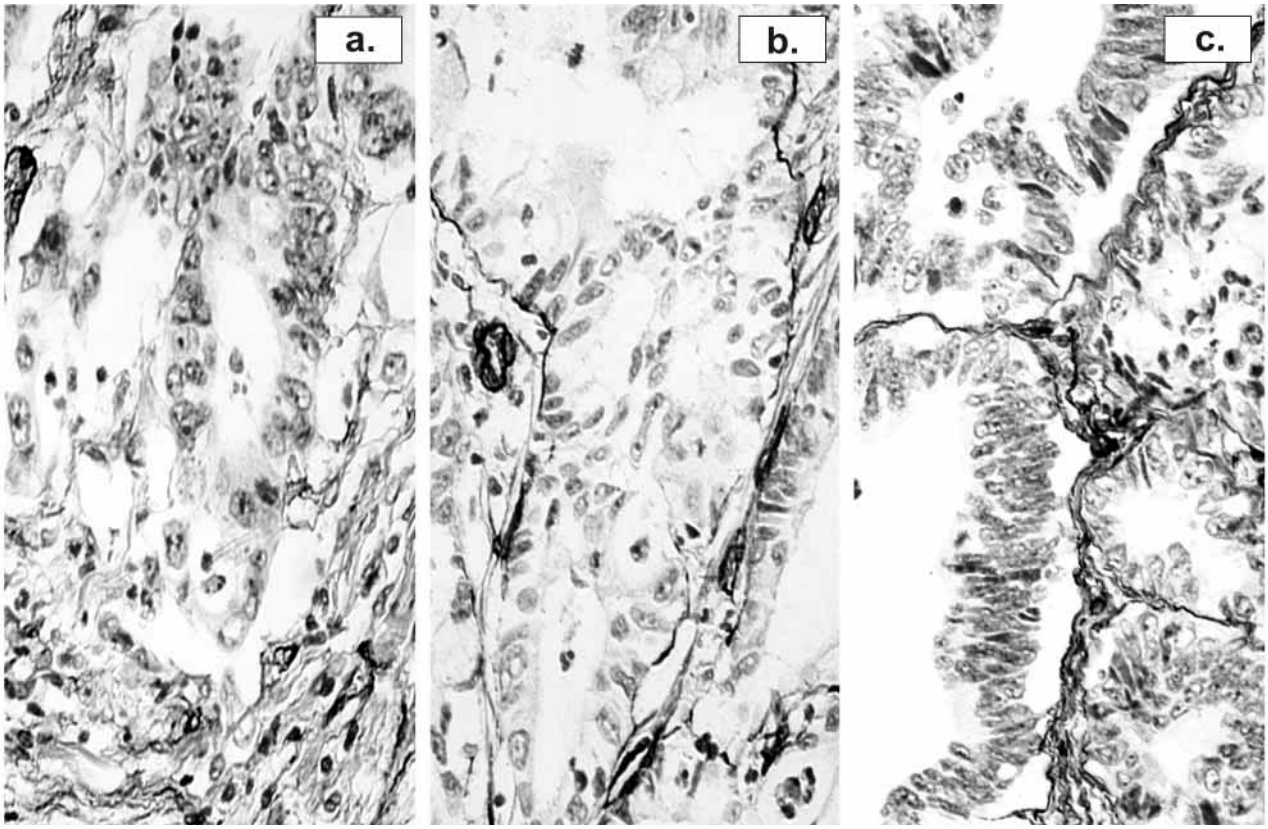


**Figure 1.** Colorectal adenocarcinoma with: mild (a) immunoreactivity to type IV collagen (collagen IV, x400), moderate (b) immunoreactivity to type IV collagen (collagen IV,x400), and severe (c) immunoreactivity to type IV collagen (collagen IV,x400)

tumor was found in 17 (42.5%), moderate in 13 (32.5%), and severe in 10 (25%) patients.

Table 1 shows the correlation between expression of collagen type IV and major clinicopathologic variables. There were no statistically significant associations between BM synthesis and sex and age of patients, and localization and histological type of CRC. Significant differences, however, existed with respect to the stage of disease and histological grade of tumor with tendency of lesser synthesis of BM in advanced stage of disease and poorer histological grade of tumor. Mean survival rate after the surgery in whole study population was 44.875±19.04 months. The 5-year survival of study population was 55%. The average overall survival in patients with mild synthesis of BM in CRC was 38.13±21.81 months in contrast with 57.36±8.74 months in patients with severe synthesis of BM in tumor tissue. The Kaplain-Meier survival curves showed statistically significant differences in the overall survival rates between patients with mild and severe synthesis of BM around CRC tumor nests; there were no statistically significant differences between survival in patients with mild and moderate and moderate and severe synthesis of BM on the invasive front of CRC (Figure 2).

**Table 1.** Description of clinicopathological data and associated distribution of collagen type IV expression counts

| Variable | Expression of collagen IV score 1 | Expression of collagen IV score 2 | Expression of collagen IV score 3 | Significance |
|---|---|---|---|---|
| Age±SD | 63.07±8.05 | 61.64±10.31 | 63.54±8.98 | NS |
| Total (n=40) | 17 (42.5%) | 13 (32.5%) | 10 (25%) | NS |
| Sex | | | | NS |
| Female (n=16) | 5 (31.25%) | 7(43.75%) | 4 (25%) | |
| Male (n=24) | 12 (50%) | 6 (25%) | 6 (25%) | |
| Localization | | | | NS |
| Right colon (n=11) | 4 (36.36%) | 6 (54.55%) | 1 (9.09%) | |
| Left colon (n=12) | 4 (33.33%) | 4 (33.33%) | 4 (33.33%) | |
| Rectum (n=17) | 9 (52.94%) | 3 (17.65%) | 5 (29.41%) | |
| Stage | | | | p=0.0376 |
| B (n=20) | 5 (25%) | 7 (35%) | 8 (40%) | |
| C (n=20) | 12 (60%) | 6 (30%) | 2 (10%) | |
| Histological type | | | | NS |
| Tubular (n=34) | 16 (47.06%) | 10 (29.41%) | 8 (23.53%) | |
| Mucinous (n=6) | 1 (16.67%) | 3 (50%) | 2 (33.33%) | |
| Histological grade | | | | p=0.0493 |
| HG-1 (n=9) | 2 (22.22%) | 2 (22.22%) | 5 (55.56%) | |
| HG-2 (n=19) | 9 (47.37%) | 7 (36.84%) | 3 (15.79%) | |
| HG-3 (n=6) | 5 (83.33%) | 1 (16.67%) | 0 (0%) | |

NS- not significant
HG-histological grade
SD-standard deviation

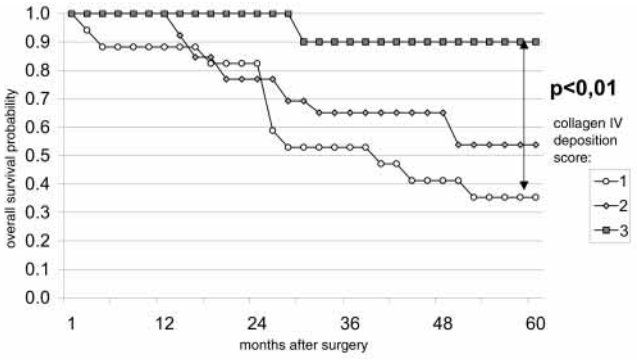


**Figure 2.** Kaplan-Meier overall survival curves of patients with colorectal cancers according to basement membrane synthesis in tumor

The estimated 5-year overall survival for CRC patients with mild and severe synthesis of BM around tumors nest was 35.29% and 90%, respectively. The univariate Cox models revealed that stage of CRC disease, histological grade of tumor and BM synthesis were prognostic factors for overall survival. In multivariate Cox analysis the stage of disease and histological grade of tumor remained independent predictors for overall survival; explicitness of BM synthesis also showed a trend towards better survival, but without statistical significance (Table 2).

**Table 2.** Prognostic influence of clinicopathological variables by univariate and multivariate Cox proportional hazard regression model for overall survival in patients with colorectal cancers with 95% confidence interval (CI) and statistical significance (P)

| Variable | Coefficient | SE | P | Hazard ratio | 95% CI |
|---|---|---|---|---|---|
| Univariate analysis | | | | | |
| Sex | 0.3242 | 0.0749 | 0.0852 | 1.38 | 1.19-1.60 |
| Localization | 0.0782 | 0.0028 | 0.9184 | 1.08 | 1.03-1.14 |
| Histological type | 0.6433 | 0.3465 | 0.0563 | 1.90 | 1.04-3.75 |
| Stage | 1.8236 | 0.4724 | 0.0101 | 6.19 | 2.45-15.64 |
| Histological grade | 1.4012 | 0.3896 | 0.0132 | 4.06 | 1.89-8.67 |
| Collagen IV deposition | 1.1887 | 0.3420 | 0.0230 | 3.28 | 1.67-6.42 |
| Multivariate analysis | | | | | |
| Stage | 1.9435 | 0.3521 | >0.001 | 6.98 | 3.50-13.92 |
| Histological grade | 1.7014 | 0.2870 | 0.032 | 5.48 | 3.12-9.62 |
| Collagen IV deposition | 1.0330 | 0.0978 | 0.064 | 2.81 | 2.32-3.40 |

CI - confidence interval
SE - standard error

## DISCUSSION

The ECM is known to play an active role in numerous biological processes such as differentiation, cell migration, proliferation, apoptosis and tumor biology. Extensive alterations of epithelial BM and of interstitial ECM occur during the progression of most invasive carcinomas. There are abundant evidences that epithelial tumor cells and stromal (mesenchymal) cells are able to produce and depose BM constituents (6,15). Collagen, which is the major component of the interstitial ECM, is primarily affected in the stromal changes at the site of tumor cell invasion (7). The implementation and differentiation of the enteroendocrine cells in the colorectal crypt include synthesis and accumulation of a collagen-IV matrix, not observed in other colorectal epithelial cells (16). The study of Pucci-Minafra describes the occurrence of an immature, oncofetal form of collagen, in breast and colon cancer. The collagen neosynthesis occurring at tumor-host interface is deeply deregulated, and therefore has to be considered as the result of altered collagen gene expression correlated with the tumor progression, rather than as a mere defensive reaction of the host cells (17). Chen et al., found that type IV collagen is intensively expressed in the interstitial stromal cells of scirrhous extrahepatic bile duct carcinoma and scirrhous gastric carcinoma, where it may play a role in desmoplastic stroma formation (18). Fisher et al., described expression of a gene COL11A1 and COL5A2, which are associated with malignancy of colon cells. These genes are not normally expressed in adult colon tissue, but were found to be expressed in 79% of examined CRC (19). In in vitro study Kim et al., suggested the importance of BM and ECM in differentiation and morphogenesis of a human colon cancer cell line. Cells cultured on plastic reconstituted BM (Matrigel) showed gland formation with highly polarized cells containing basal nuclei, while, on the other hand, cells cultured without presence of BM were poorly differentiated and contained a few glandular structures with small lumens (20). There is a close relationship between cellular phenotype and specialized ECM formation. Cell synthesis of ECM, according to their state of maturation and function, and on the other hand matrix components are able to modulate fundamental cellular properties. Collagen can influence polarity, proliferation and differentiation of epithelial cells (21). Specific (integrins) receptors for collagens and the ECM proteins have been identified on various normal and transformed cells. These receptors may mediate the effects of collagens on cell proliferation and differentiation by acting as transducers of signals between the collagen matrix and the cytoskeleton (22). Zeng et al. demonstrated that loss of BM type IV collagen is involved in human colorectal tumorigenesis. Despite extensive architectural disorganization benign tumor retains continuous type IV collagen staining in the BM. In contrast, focal defects in BM type IV collagen staining are apparent in lesions of carcinoma in situ. Furthermore, invasive CRC demonstrates zones devoid of type IV collagen staining around invasive tumor cells (5). Proteolytic degradation of ECM is a critical event during tumor invasion, angiogenesis and metastasis. By double immunostaining, matrix metalloproteinase-9 expression was observed to localize within areas of limited type IV collagen staining. Similarly, type IV collagen staining was observed to be greatest in areas devoid of matrix metalloproteinase-9 expression (5).

In the majority of literature references a loss of the continuous linear deposit of BM around invasive carcinomas has been suggested to correlate with their increased invasiveness. In contrast, extensive BM deposition would represent a more favorable prognosis but there is experimental evidence that some transplantable neoplasms with ability to synthesize and deposit large amounts of BM components after subcutaneous inoculation give the presence of numerous metastatic foci also surrounded by ECM. These results cast doubt on the hypothesis that the extensiveness of BM deposition might always be of prognostic value by being inversely correlated with the degree of invasiveness of a carcinoma (23).

In the light of the significance of BM synthesis in CRC previous literature results are, in part, contradictory. In our work we demonstrated statistically significantly explicit synthesis of BM in B vs. C stage of CRC, and in tumor with higher histological differentia-

tion. Patients with severe synthesis of BM had significantly longer overall survival in contrast to patients with mild expression of BM. These data confirm some but not all previous reports: Havenith et al., using antibodies to type IV collagen, BM deposition studied in 163 cases of CRC. Cases with limited BM deposition showed an overall significant shorter survival and were overexpressed in Dukes' stages C and D. Even extent of BM deposition in Dukes' stage C had prognostic value (24). Inada et al. investigated the development of hepatic metastases in 344 CRC patients for correlation with the presence of both venous invasion and BM deposition in the tumor tissue. BM deposition was seen more frequently in Dukes' A tumors than in B tumors although this was not statistically significant. No relationship was found between BM positivity and five-year survival. Deposition of BM was dependent on the grade of tumor differentiation whereas it had no direct relation to the development of liver metastasis (25). Offerhause et al., in 154 CRC specimens, using antibody collagen IV, in contrast to our study, did not find significant association between collagen IV expression and Dukes' classification, grade of tumor, number of positive lymph nodes or invasion of blood vessels. Collagen IV expression was prognostic factor only by univariate Cox proportional hazard model (4). Knežević-Ušaj evaluated 81 patients with CRC and used laminin for visualization of BM. Extent of laminin positivity was significantly different between Dukes' A vs. C and D stages while no significant differences were found between B vs. C and D stages. There were not correlations between production of laminin and histological and macroscopical type of tumor, histological and nuclear grade of tumor tissue. Survival in patients with low expression of laminin was significantly shorter than in patients with extensive production of laminin (26). Some literature data suggest that specification of distinct type IV collagen alpha chains has grater prognostic significance. Hiki et al., studied immunohistochemical localization of six genetically distinct type IV collagen alpha chains of BM. In the normal colorectal mucosa and in the tubular adenomas alpha1/alpha2(IV) and alpha5/alpha6(IV) chains were stained in all epithelial BM. However, in intramucosal carcinomas, the assembly of alpha5/alpha6(IV) chains into the BM was inhibited in a discontinuous or negatively stained pattern. These authors suggested that differential immunohistochemical localization of the type IV collagen alpha5/alpha6 chains could be one diagnostic marker for the invasiveness of CRC (27). Ban et al. studied the immunohistochemical localization of integrins (alpha2, alpha6) and BM components (type IV collagen, laminin) in colorectal adenomas and adenocarcinomas. The basal polarization of alpha6 integrin was connected with well preserved BM seen in adenomas, intramucosal carcinomas and in well-differentiated areas of invasive CRC while in moderately and poorly differentiated areas the tumor cells showed diffuse expression of alpha6 integrin connected with disorganization or loss of the BM components (28). Some authors report importance of determination of various types of collagen in BM. Hilska et al., emphasized the importance of immunolabeling for type I and III collagens adjacent to the BM and cancerous stroma with conclusion that both the epithelial BM and the collagenous matrix immediately beneath it are degraded in malignant tissue by the simultaneous activation of several degradative enzymes or alterations in the expression of collagen subtypes in malignant tissue (29). In his article Visser gives new views on significance of the distribution of type VII collagen in the BM. In the normal colonic mucosa type VII collagen was not observed. In adenomas of the colon, type VII collagen staining was detected only in connection with dysplastic epithelium. In the invasive CRC type VII collagen staining was detected only in well or moderately differentiated carcinomas in higher amounts. These findings therefore reveal a transient expression of type VII collagen in the transition of dysplastic epithelium into carcinoma suggesting the involvement of type VII collagen in the process of early invasion (30). Amenta et al. characterized the new group of nonfibrillar collagens localized to the BM zone, type XV collagen, with possible functions to adhere BM to the underlying stroma. In moderately differentiated invasive adenocarcinomas type XV collagen was characterized as virtually absent from the BM zones of malignant glandular elements, beside continuous or often discontinuous deposits of laminin and type IV collagen. Nevertheless there were also similarities; all 3 proteins were usually present in the stroma and adjacent vascular BM zones surrounding invasive glands. The loss of type XV collagen from malignant epithelial BM zones and its increased interstitial expression suggests a role for this protein in the invasive process and the possibility that it may provide a sensitive indicator of tumor invasion (31).

## CONCLUSION

There is strong evidence that neoplastic cells can produce BM components. These components may be characteristic of the tumor, may reflect its biology and may be useful in predicting patient survival. The BM synthesis would be valuable as a prognostic factor if it provided information that supplemented conventional staining parameters. Immunohistochemical staining for collagen IV is an important additional technique with prognostic value.

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