Prediction of Occult Neck Metastases in Laryngeal Carcinoma: Role of Proliferating Cell Nuclear Antigen, MIB-1, and E-Cadherin Immunohistochemical Determination

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ABSTRACT

The aim of this study is to investigate the predictive value of proliferative activity assessment and E-cadherin expression by means of immunohistochemistry in identifying patients with laryngeal squamous cell carcinoma at a high risk for occult node metastasis. Thirty consecutive patients treated for laryngeal carcinoma with clinically negative nodes (occult metastases, pN+) between the years 1980 and 1990 were selected for this study. A group of 30 cases with negative cervical lymph nodes (pN−) having a similar anatomic site and tumor size distribution was used as control. In each case, several histological parameters, including grade, pattern of invasion, number of mitosis (×10 high-power field), tumor inflammatory infiltrate, and tumor sclerosis, were assessed. Proliferative activity was determined using immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and MIB-1. Other putative prognostic factors investigated at the immunohistochemical level were the cell adhesion molecule E-cadherin and two oncoproteins, p53 and c-erbB-2. In pN+ cases, the expression of PCNA and MIB-1 was significantly higher than in the pN− group. Moreover, a significant loss of E-cadherin expression was observed in carcinomas with occult metastases. No differences in p53 and c-erbB-2 oncoproteins were found between pN+ and pN− cases. Among the other pathological parameters examined, only histological grade was significantly associated with the presence of occult metastases, but on multivariate analysis, this relationship was lost. We conclude that PCNA, MIB-1, and E-cadherin are independent predictors of occult nodal disease in laryngeal squamous cell carcinoma, and their immunohistochemical determination could be useful in identifying patients with clinically negative lymph nodes who are at considerable risk for occult metastases and who may benefit from elective neck dissection.

INTRODUCTION

Preoperative identification of occult lymph node metastasis represents a crucial point in the clinical management of patients with laryngeal cancer for its prognostic and therapeutic implications (1). Data from literature indicate that 4–40% of patients with laryngeal carcinoma and clinically negative neck lymph nodes have indeed occult metastases on subsequent histological examination (2–6). As a consequence, treatment of N0 patients is a matter of great controversy between those who are in favor of a "wait and see" attitude and those who prefer elective neck dissection. Indeed, prophylactic neck dissection eliminates the risk of late metastases in N0 cases, but exposes the patient to a higher morbidity and higher hospital costs, which are in most cases unnecessary (1, 3). Another possible disadvantage of lymphadenectomy may be the local reduction of the immunological host defense, which may facilitate the spread of residual tumor cells. Therefore, the identification of biological tumor factors indicative of high or low risk of occult lymph node metastases would be extremely helpful in deciding on elective or delayed therapeutic strategy in the treatment of the neck in N0, laryngeal cancer.

Previous clinicopathological studies have shown that the presence of locoregional metastases in patients with laryngeal carcinoma is significantly associated with some histological features of the primary tumor, such as the degree of differentiation, tumor growth pattern, presence of peritumoral desmoplasia, and inflammation (7–9). Additional studies confirmed that histological grade and host immune response correlate significantly with occult lymph node metastasis (1, 10). However, because of the absence of established criteria, the evaluation of this group of histological parameters is very subjective, and reproducibility is poor.

In recent years, the efforts of several research groups have led to a dramatic improvement in the understanding of the neoplastic growth process and have furnished novel molecular tools that may help in identifying tumors more likely to metastasize, being therefore potentially useful for management and prognostic purposes. In particular, several lines of evidence suggest that the evaluation of tumor cell proliferative activity, cell adhesion markers, and oncoprotein expression could allow an estimate of the biological behavior of head and neck squamous cell carcinoma. When proliferative activity was evaluated by tumor labeling index in a series of head and neck squamous cell carcinomas, the results indicated that lesions with a high proliferative activity have a poorer survival (11). Recently, Benazzo et al. observed a significant correlation between proliferative indices measured after in vivo bromodeoxyuridine incorporation and tumor dimension, histological differentiation, and lymph node involvement in a group of patients with carcinomas of the head and neck (12). Similar results were obtained...
when proliferation was measured by flow cytometric analysis of DNA content (13).

Loss of intercellular adhesion might be an important event in the early phases of the metastatic process. The potential relationship between the expression of the adhesion molecule E-cadherin, a member of a family of calcium-dependent cell-cell adhesion molecules also including P- and N-cadherin, and the presence of lymph node metastasis in laryngeal carcinoma has been the object of studies by different groups. Schipper et al. observed that E-cadherin expression decreased with loss of differentiation in primary carcinomas, and that lymph node metastases expressed a lower level of the protein, suggesting an important role of cadherin loss in the metastatic process (14). In a subsequent study, Mattijssen et al. were unable to find any significant relationship between the level of E-cadherin expression and the presence of lymph node metastasis in a group of 50 head and neck squamous cell carcinomas (15). However, these studies only examined patients with clinical evidence of metastatic disease, and no information is available in the literature about the relationship between E-cadherin expression and the presence of occult lymph node metastases.

Indeed, most of the studies concerning predictors of the behavior of carcinomas of the head and neck have considered heterogeneous groups of lesions concerning both the site of primary lesion and the clinical node status. The present study is focused on a homogeneous population of patients with laryngeal squamous cell carcinoma and clinically negative neck lymph nodes with the aim of defining prognostic parameters that permit the identification of patients at risk for occult metastases. We evaluated several histopathological features of primary lesions, and determined immunohistochemically tumor cell proliferative activity and E-cadherin expression. Moreover, we studied the expression of c-erbB-2 and p53 oncoproteins, the products of two genes known to be involved in the development of head and neck squamous cell carcinomas (16–18).

### PATIENTS AND METHODS

**Cases Studied.** Thirty consecutive patients treated at the Institute of Otorhinolaryngology of the University of Florence between 1980 and 1990 for laryngeal squamous cell carcinoma with occult laterocervical metastases were selected for this study. All patients had clinically negative laterocervical lymph nodes and underwent elective neck dissection, with subsequent histological recognition of one or more occult metastases (pN+). A group of 30 patients treated in the same period for laryngeal squamous cell carcinoma with clinically and histologically negative laterocervical lymph nodes (pN−) was used as control. Clinical data of the two groups of patients are summarized in Table 1.

<table>
<thead>
<tr>
<th>Site</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>Partial</th>
<th>Total</th>
<th>Age (Mean)</th>
<th>Sex M:F</th>
</tr>
</thead>
<tbody>
<tr>
<td>pN+</td>
<td>20</td>
<td>10</td>
<td>7</td>
<td>8</td>
<td>10</td>
<td>5</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>pN−</td>
<td>20</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>8</td>
<td>5</td>
<td>16</td>
<td>14</td>
</tr>
</tbody>
</table>

*p TNM staging.

Formalin-fixed, paraffin-embedded tissue samples from resection specimens were retrieved in each case, and serial sections were stained with H&E or used for the immunohistochemical studies. In each case, the following histological parameters were assessed: histological degree of differentiation (well, moderately, or poorly differentiated), pattern of invasion (pushing or infiltrating), number of mitoses (×10 high-power field), peritumoral inflammatory infiltrate (present or absent), and peritumoral sclerosis (present or absent).

**Immunohistochemistry.** Immunohistochemical studies were performed by the avidin-biotin complex technique (Dakopatts, High Wycombe, UK). Proliferative activity was investigated using monoclonal antibodies against Ki-67 (M1B-1, Immunotech, Marseille, France) and PCNA (PC-10, DAKO Corp., Carpenteria, CA). For E-cadherin detection, the monoclonal antibody HECD-1 (Nuclear Laser Medicine, Milan, Italy) was employed. For p53 and c-erbB-2 detection, the monoclonal antibodies DO7 (DAKO Corp.) and mAb1 (Triton Diagnostics, Alameda, CA) were used. MIB-1, E-cadherin, and p53 immunostaining required microwave pretreatment of dewaxed and rebred sections. Negative controls were performed by substitution of the primary antibody with nonimmune rabbit or mouse serum.

Scoring of the immunostaining was performed by one observer (A. F.), who had no knowledge of patients' clinical status, using a standard light microscope equipped with an eyepiece grid of 10 × 10 squares and a ×40 objective. In each case, at least 1000 tumor cells were counted from areas with high and low expression of the antigen. All stained cells were considered positive regardless of the intensity of the staining, and the results were expressed in percentage of positive cells. An arbitrary cutoff value of 20% positive cells was chosen to separate cases with high and low expression of the antigens.

**Statistical Analysis.** Statistical tests were performed using EGRET (Statistics and Epidemiology Research Corporation, Seattle, WA) and Stata (Stata Corporation, College Station, TX). The relationship between clinical and pathological variables was assayed by Fisher's exact test. The correlation between the different immunohistochemical indices was evaluated using Spearman's correlation coefficient. The role of each possible prognostic factor (univariate analysis) and the joint effect of all these factors (multivariate analysis) was explored using the multivariate logistic regression analysis. The final results of these analysis are the ORs and their 95% CIs. The likelihood

2 The abbreviations used are: PCNA, proliferating cell nuclear antigen; OR, odds ratio; CI, confidence interval.
The percentage of E-cadherin-positive cells decreased in seven cases, and four of these were poorly differentiated carcinomas of the larynx (Fig. 3). Cytoplasmic staining was observed in most tumor cells, immunoreactivity was associated with the cytoplasmic membrane (Fig. 3). Diffuse nuclear immunostaining for PCNA in a poorly differentiated squamous cell carcinoma.

**RESULTS**

Table 2 synoptically summarizes the results of the immunohistochemical and histopathological analysis of the pN+ and pN− groups.

All 60 tumors stained positively for PCNA, which localized in the nucleus, with different degrees of intensity (Fig. 1). Some of the mitotic figures were associated with weak and diffuse cytoplasmic staining. The PCNA score for the whole series ranged between 6.4 and 71.7% (25.6 ± 15.9, mean ± SD). Twenty-four patients in the pN+ group had a tumor with PCNA score greater than 20%, whereas only six patients of the pN− group had carcinomas with more than 20% of positive cells (P < 0.001; Fisher’s exact test).

Positivity for MIB-1 was observed in all cases. Only nuclear staining having a finely granular pattern was observed (Fig. 2). Almost all mitotic figures were stained. The percentage of positive cells ranged between 2 and 49.1% (mean, 18.9 ± 10.3). In the pN+ group, 18 cases showed more than 20% of positive cells, whereas in the pN− group, only 5 cases had a MIB-1 score >20% (P < 0.001; Fisher’s exact test).

Considering the whole series of 60 cases, the percentage of labeled cells tended to be higher using the PCNA antibody. However, a significant correlation between PCNA and MIB-1 immunoreactivity in each case was found (r_s = 0.61; P < 0.001). Both PCNA and MIB-1 scores increased as tumors became less differentiated (P = 0.03 and 0.2, respectively; Fisher’s exact test). No relationship was found between PCNA and MIB-1 indices and mitosis count.

E-cadherin expression varied greatly from case to case, and in most tumor cells, immunoreactivity was associated with the cell membrane (Fig. 3). Cytoplasmic staining was observed in seven cases, and four of these were poorly differentiated carcinomas. The percentage of E-cadherin-positive cells decreased significantly in the less-differentiated tumors (P = 0.001; Fisher’s exact test). Moreover, a significant association was found between low levels of cadherin expression (≤20% of positive cells) and the presence of occult lymph node metastasis (P = 0.006; Fisher’s exact test). When the expression of proliferation markers (PCNA and MIB-1) was compared with E-cadherin expression in each single case of the whole series, no significant relationship was found. However, when groups with high and low expression of the antigens (>20% and ≤20%) were compared, it became evident that cases with high proliferative activity tended to have a low expression of E-cadherin. In particular, a significant relationship was found between high expression of PCNA and low expression of E-cadherin (P = 0.004; Fisher’s exact test).

Regarding p53 expression, 39 cases (65%) showed positive nuclear staining for the oncoprotein (Fig. 4). No significant differences were found in p53 levels between the pN+ and pN− group using either a four-point scale scoring system (−, negative; +, +1–10%; ++, 11–50%; and ++++, >50%; Ref. 19) or a single cutoff point (≤20% or >20% positive cells). No relationship was evidenced between p53 expression and proliferation indices. C-erbB-2 oncoprotein was detected in 17 cases (28.3%). In 14 instances, the immunostaining localized at the cytoplasmic membrane level (Fig. 5), and in 3 there was diffuse cytoplasmic staining. Six cases from the pN+ group and two cases from the pN− group showed a number of positive cells greater than 20%, but this difference was not significant. Similarly, no significant differences were found using a three-point scale scoring system (−, negative; +, ±50%; and ++, >50%; Ref. 20).

The distribution of percentages of positively stained cells...
using the antibodies against PCNA, MIB-1, E-cadherin, p53, and c-erbB-2 in each individual tumor of pN− and pN+ groups is reported in Fig. 6.

Among the several histopathological features analyzed, only the histological grade had a significant relationship with the presence of occult metastases ($P = 0.02$; Fisher’s exact test), as patients with poorly differentiated carcinomas had a higher risk of occult metastatic disease.

Univariate logistic regression analysis of the different parameters showed that high levels of PCNA and MIB-1, low levels of E-cadherin, and poor histological differentiation were significantly associated with the presence of occult lymph node metastases. On multivariate analysis, only PCNA, MIB-1, and E-cadherin expression maintained an independent status for the prediction of occult lymph node metastases (Table 3).

Comparison of the immunohistochemical findings in $T_3-T_4$ tumors versus $T_1-T_2$ tumors showed that the advanced lesions had a higher proliferative activity as assessed with both PCNA and MIB-1 stainings ($P = 0.009$ and $P = 0.07$, respectively; Fisher’s exact test) and decreased E-cadherin expression ($P = 0.07$; Fisher’s exact test). Levels of p53 and c-erbB-2 expression were comparable in the two groups. No significant difference for any of the markers tested was evidenced between glottic and supraglottic carcinomas.

**DISCUSSION**

The metastatic process is the result of the progressive acquisition of an aggressive tumor cell behavior, characterized by increased cell proliferation rates, modification of cell adhesive properties, and genetic alterations. Accordingly, in the present study, we observed that in laryngeal squamous cell carcinoma, a high proliferative activity assessed by MIB-1 and PCNA immunostaining, and low expression of the adhesion molecule E-cadherin, significantly correlated with the presence of occult nodal metastases.

Until recently, a major limitation in evaluating the prognostic significance of these markers by means of immunohistochemistry, which allows the in situ identification of the antigen, was the necessity of employing fresh tissue, in which morphology preservation is often suboptimal, thus precluding the use of archive material. This problem has been overcome partially by the introduction of methods of antigen retrieval, such as microwave oven pretreatment of paraffin sections, which have greatly facilitated the study of putative prognostic markers in surgical pathology of tumors (21). The reliability of this technique in studying cell proliferation and cell adhesion processes has been demonstrated clearly by several groups (21–23). A double assessment of proliferative activity using both PCNA and MIB-1 immunostainings was performed in this study, because it has been pointed out that PCNA immunostaining may suffer from some limitations, the major one being a reduction of positivity when fixation time exceeds 48 h (24). On the other hand, MIB-1 determination seems not to be affected by fixation time (25), but experimental data suggest that reduction of blood supply, which is a common event in central areas of a tumor, may determine a modification of the expression of the antigen (26). In the present series, PCNA index tended to be higher than MIB-1 index. Nevertheless, a significant relationship was found between the expression of the two antigens as already observed in other malignancies (27–29). On the other hand, no relationship was found between the expression of PCNA and MIB-1 indices and mitosis count, possibly because these parameters evaluate different phases of the proliferating cell cycle.

The results of the present study indicate that tumor cell proliferation indices assessed by immunohistochemistry correlate strongly with the presence of occult metastases in laryngeal squamous cell carcinoma. A similar relationship has been found previously in carcinomas from different sites (28, 30). Several studies have now confirmed the adverse prognosis of laryngeal carcinomas with high proliferative activity as well as the association of other prognostic parameters, such as the degree of differentiation and ploidy status, with enhanced proliferation (11, 31). Therefore, enhanced proliferative activity may not only be important in the early phases of malignant transformation of the squamous epithelium of the larynx (32, 33) during the transition from dysplasia to invasive carcinoma but could also be a feature of lesions with a higher tendency to spread and metastasize. Our findings are discordant with those of a recent study, in which no association was found between PCNA and Ki-67 indices and the presence of lymph node metastasis in a series of laryngeal carcinomas (34). A comparison of the results is difficult, because the distribution of PCNA and Ki-67 indices in the node-negative and node-positive groups is not indicated in this article. In addition, the carcinomas examined belonged to different histotypes (squamous cell, spindle cell, large cell undifferentiated, and neuroendocrine), and patients had different clinical N status, whereas we considered only squamous cell carcinomas in patients with clinically negative neck lymph nodes.

Fig. 2 MIB-1 nuclear immunoreactivity in a well-differentiated squamous cell carcinoma. The staining is distributed predominantly at the periphery of tumor nests.
Sensitivity (35, 36). Our results confirm that there is a significant loss of E-cadherin expression in poorly differentiated squamous cell carcinomas of the larynx (14, 15). Moreover, we observed that loss of tumor differentiation was also accompanied by

Fig. 3 E-cadherin expression in a well-differentiated squamous cell carcinoma. The immunostaining is localized at the cell membrane of most tumor cells (A). In a moderately differentiated carcinoma, only a few tumor cells express the antigen (B).

Fig. 4 p53 oncoprotein expression in a moderately differentiated squamous cell carcinoma.

Several recent studies have shown that E-cadherin expression is reduced in epithelial tumors from different sites when compared with the epithelia of origin; these modifications have been correlated with tumor degree of differentiation and aggressiveness (35, 36). Our results confirm that there is a significant loss of E-cadherin expression in poorly differentiated squamous cell carcinomas of the larynx (14, 15). Moreover, we observed that loss of tumor differentiation was also accompanied by

Fig. 5 c-erbB-2 immunostaining in a well-differentiated squamous cell carcinoma.
Predictors of Occult Metastases in Laryngeal Carcinoma

**Table 3** Univariate and multivariate logistic regression analysis of 60 laryngeal cancer patients according to presence (pN+) or absence (pN−) of occult neck metastases

<table>
<thead>
<tr>
<th>Prognostic parameter</th>
<th>Categories</th>
<th>Univariate analysis</th>
<th>Multivariate stepwise analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td>LRS (df)</td>
</tr>
<tr>
<td>PCNA</td>
<td>≤20%</td>
<td>1.00</td>
<td>16.00 (4.52-56.70)</td>
</tr>
<tr>
<td></td>
<td>&gt;20%</td>
<td>1.00</td>
<td>8.64 (2.57-29.01)</td>
</tr>
<tr>
<td>MIB-1</td>
<td>≤20%</td>
<td>1.00</td>
<td>0.15 (0.04-0.55)</td>
</tr>
<tr>
<td></td>
<td>&gt;20%</td>
<td>1.00</td>
<td>0.52 (0.12-2.37)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Negative</td>
<td>1.00</td>
<td>0.68 (0.12-3.82)</td>
</tr>
<tr>
<td></td>
<td>≤50%</td>
<td>1.00</td>
<td>0.66 (0.19-2.31)</td>
</tr>
<tr>
<td></td>
<td>&gt;50%</td>
<td>1.00</td>
<td>1.45 (0.36-5.94)</td>
</tr>
<tr>
<td></td>
<td>Histological grade</td>
<td>1.00</td>
<td>2.08 (0.60-7.24)</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>1.00</td>
<td>0.58 (0.21-1.62)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1.00</td>
<td>0.87 (0.31-2.46)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Number of mitoses</td>
<td>≤10'</td>
<td>1.00</td>
<td>0.58 (0.21-1.62)</td>
</tr>
<tr>
<td></td>
<td>&gt;10</td>
<td>1.00</td>
<td>0.87 (0.31-2.46)</td>
</tr>
<tr>
<td>Peritumoral inflammation</td>
<td>Absent</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>1.00</td>
<td>0.58 (0.21-1.62)</td>
</tr>
<tr>
<td>Peritumoral sclerosis</td>
<td>Absent</td>
<td>1.00</td>
<td>0.87 (0.31-2.46)</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Computed by likelihood ratio statistic; df, degrees of freedom.

*Adjusted OR of metastasis.

*Reference category.
cytoplasmic localization of the antigen in a number of cases, thus confirming the findings obtained in other carcinomas and adenocarcinomas (37–39). These data taken together suggest that deregulated E-cadherin expression is related to the acquisition of a dedifferentiated and more aggressive phenotype by the neoplastic cells, resulting in a higher tendency to spread to locoregional lymph nodes (36). In contrast to our data, Mattijsen et al. found no relationship between the levels of E-cadherin expression and the presence of lymph node metastases in a group of 50 head and neck squamous cell carcinomas (15). However, these authors included in their series squamous cell carcinomas from different sites, thus limiting a direct comparison of the results.

In vitro studies have shown that cell-cell adhesion is related strictly to proliferation and differentiation processes. Decreased proliferative activity has been noted when stratification is induced in cultures of normal human urothelial cells, a process that is associated with desmosome formation and increased expression of E-cadherin (40). Similarly, cyclic AMP and relaxin induce differentiation, growth inhibition, and increased expression of E-cadherin in cultured human breast cancer cells (41, 42). We observed that loss of cadherin expression was accompanied by an increased PCNA and MIB-1 expression in adenocarcinomas (37-39). These data taken together suggest that is associated with desmosome formation and increased expression of E-cadherin (40). Similarly, cyclic AMP and relaxin induce differentiation, growth inhibition, and increased expression of E-cadherin in cultured human breast cancer cells. We observed that loss of cadherin expression was accompanied by an increased PCNA and MIB-1 expression in adenocarcinomas (37-39). These data taken together suggest that the use of an immunohistochemical determination of tumor cell proliferation and E-cadherin expression in specimens of laryngeal squamous cell carcinoma may help in the identification of those patients with cervical lymph node metastases from epidermoid carcinoma of the larynx and their relationship to certain characteristics of the primary tumor: Cancer (Phila.), 14: 55–66, 1960.

The evaluation of several traditional histopathological parameters showed that only the degree of differentiation was correlated significantly with the presence of occult lymph node metastases in our series. Moreover, we prevalently obtained membrane immunostaining, whereas the above-mentioned authors observed only cytoplasmic staining. As observed previously, the study of the expression of p53 and c-erbB-2 oncoproteins failed to reveal any significant relationship with the presence of lymph node metastases in laryngeal squamous cell carcinoma (17, 34, 45).

The evaluation of several traditional histopathological parameters showed that only the degree of differentiation was correlated significantly with the presence of occult lymph node metastases in our series. However, in multivariate analysis, this parameter failed to add independent information to identify patients at risk for occult disease. The absence of prognostic significance of parameters such as tumor growth pattern, peritumoral sclerosis, and inflammatory reaction, which has been correlated previously with lymph node status in laryngeal carcinoma by other authors (1, 7, 10), points out the difficulty in obtaining an objective and reproducible evaluation of these histopathological features.

In conclusion, our study indicates that the use of an immunohistochemical determination of tumor cell proliferation and E-cadherin expression in specimens of laryngeal squamous cell carcinoma may help in the identification of those patients with clinically negative lymph nodes who are at considerable risk for occult metastases. Although the sensitivity and specificity of these markers may still be insufficient to allow a decision of elective neck dissection, they may supplement the results obtained by sophisticated imaging techniques and be incorporated in a multidisciplinary approach to the definition of the appropriate therapeutic strategy in the treatment of the neck in these patients.

REFERENCES


Prediction of occult neck metastases in laryngeal carcinoma: role of proliferating cell nuclear antigen, MIB-1, and E-cadherin immunohistochemical determination.

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