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Expression of E-cadherin in squamous cell carcinomas of the supraglottic larynx with correlations to clinicopathological features

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Abstract

The aim of this study was to investigate the prognostic significance of E-cadherin expression in squamous cell carcinomas of the supraglottic larynx. 101 primary carcinomas were retrospectively studied. The level of E-cadherin expression was determined by immunohistochemistry. There was a significant correlation between decreased E-cadherin expression and the presence of nodal metastases ($P=0.007$). T-stage ($P=0.025$) and histological grade ($P=0.043$) were also associated with nodal metastases. Multivariate analysis confirmed that these three parameters were independent predictors of nodal metastases. Decreased E-cadherin expression also correlated with an increase in recurrence rates ($P=0.019$). However, in multivariate analysis only pathological N-stage was significantly associated with disease-specific survival. We conclude that E-cadherin is an independent predictor of nodal metastases in supraglottic squamous cell carcinomas. Determination of E-cadherin expression levels might be useful in identifying patients with clinically negative lymph nodes who are at risk of occult metastases, allowing more effective treatment strategies to be implemented.

Keywords: Squamous cell carcinoma; Head and neck; E-Cadherin; Adhesion molecules; Prognosis

1. Introduction

It has long been known that cell–cell adhesion is commonly reduced in human cancers. Reduced cell–cell adhesiveness is associated with loss of contact inhibition of proliferation, thereby allowing escape from growth control signals. Moreover, the suppression of cell–cell adhesiveness may trigger the release of cancer cells from the primary cancer nests and confer invasive properties on a tumour. Therefore, reduced cell–cell adhesiveness is considered indispensable for both early and late carcinogenetic steps [1].

In epithelial tissues, cell–cell adhesion is mediated largely by the members of the cadherin family and in particular by E-cadherin. Cadherins are a family of calcium-dependent cell adhesion molecules, which mediate predominantly homotypic cell–cell interactions and play a key role during morphogenesis, as well as in the maintenance of the differentiated phenotype. They are transmembrane glycoproteins with a highly conserved cytoplasmic tail, which interacts with the cytoskeleton via the intracellular proteins α, β and γ catenins. The cadherin family contains several members, depending on their tissue distribution, including E-cadherin, which forms the key functional component of adherens junctions between epithelial cells [1,2].

Many studies documenting loss or reduction of E-cadherin protein expression have been reported in various human cancers, including squamous cell carcinomas of the head and neck (reviewed in Refs. [1,2]. Generally, E-cadherin expression was found to be strong in well-differentiated cancers, which maintain their cell–cell adhesiveness and are less invasive, but

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reduced in undifferentiated cancers, which have lost their cell–cell adhesion and show a strong invasive tendency. Therefore, inactivation of the E-cadherin-mediated invasion suppressor system was considered to result from reduced expression of E-cadherin.

In addition, significant correlations between abnormalities of E-cadherin expression and the clinical outcome of patients with cancers have been reported [2]. In squamous cell carcinomas of the head and neck, several studies have reported that abnormal expression of E-cadherin correlates with clinicopathological characteristics of the tumour, such as lymph node metastases [3–5], and disease-free survival [6]. However, a number of other studies have failed to show a relationship between E-cadherin expression and these clinicopathological variables [7–9].

In an attempt to shed more light on this subject, we have examined samples from 101 patients with squamous cell carcinoma of a single localisation, the supraglottic larynx, for E-cadherin expression using immunohistochemical techniques. The prognostic implications of changes in expression of the E-cadherin protein are discussed in relation to other prognostic indicators and patient outcome.

2. Patients and methods

2.1. Patients

101 patients with squamous cell carcinoma of the supraglottic larynx were selected from the pathological files of the Hospital Central de Asturias and the Hospital Valle del Nalón. Patients who were chosen for this study were treated between 1988 and 1994. Only male patients were included due to the exceptionally low proportion of women with this kind of tumour in our area. All the patients included in our study underwent surgical resection of their tumour and bilateral neck dissection (functional or radical based on surgical findings). All of them had a single primary tumour, none had undergone treatment prior to surgery, and had microscopically clear surgical margins. None of the patients was thought to have had distant metastases at the time of surgery. A total of 49 (49%) patients received postoperative radiotherapy. As a general rule, this was administered to the patients with histologically N2 or N3 neck lesions, and also in N0-N1 neck lesions with locally advanced stage (T4). The clinicopathological data from the patients are shown in Table 1. The stage of disease was determined after the surgical resection of the tumour according to the TNM system of the International Union Against Cancer (4th edn). The histological grade was determined according to the degree of differentiation of the tumour (Broders' classification).

2.2. Immunohistochemical study

The formalin-fixed paraffin-embedded tissues were cut into 4 μm sections and dried on capillary-gap glass slides (ChemMate, BioTEK Solutions, Santa Barbara, CA, USA). The sections were deparaffinised with standard xylene and hydrated through graded alcohols into water. Antigen retrieval procedure was performed using citrate buffer and heating for 10 min in a pressure cooker. Staining was done at room temperature on an automatic staining workstation (TechMate 1000, BioTEK Solutions) by using the Envision peroxidase mouse system (Envision Plus, Dako, Carpinteria, CA, USA). Slides were placed for 15 min into a 3% hydrogen peroxide blocking medium and then allowed to react with the primary antibody, the anti-E-cadherin antibody (clone 36, Transduction Labs., Lexington, KY, USA), at a concentration of 1:2000 for 30 min. Immunodetection was performed with the Envision system and chromogen as a substrate. Counterstaining with haematoxylin for 1 min was the final step. Following staining, the slides were dehydrated through graded alcohols and mounted with a coverslip using a standard medium. Appropriate positive controls were used

Table 1
Characteristics of the patient population and their tumours

Characteristic	Patients *n*
Mean age at resection (median)	
Total population: 62 (63) years	
PT stage	
T1	13
T2	38
T3	37
T4	13
PN stage	
N0	46
N1	19
N2	31
N3	5
Disease stage	
I	8
II	22
III	30
IV	41
Histological grade	
Well differentiated	45
Moderately differentiated	36
Poorly differentiated	20
Outcome	
Alive at last follow-up	51
Died of index cancer	29
Died of other causes	21
Mean length of follow-up (median)	
Total population: 44.6 (37) months	
Alive at last follow-up: 61.2 (61) months	
Died of other causes: 36 (22) months	
Died of index cancer: 19.9 (12) months	

(normal laryngeal epithelium). Negative controls with an omission of the antiserum from the primary incubation were also included.

The slides were viewed randomly, without clinical data, by one of the authors. The staining was predominantly membranous with some cytoplasmatic staining. A staining score was given based on the intensity of the membranous stain (0–4) and percentage of cells stained (0–100%). The two components were multiplied for an overall staining score between 0 and 400. A score was given for both the tumour and the surrounding normal-appearing epithelium.

2.3. Statistical analysis

Staining scores were averaged for each group, and mean scores were compared within groups stratified with respect to clinicopathological parameters (T-stage, N-stage, degree of differentiation and relapse) and within the overall group. For this comparison, we used the one-way ANOVA test. In addition, T-stage and degree of differentiation were related to the nodal status in contingency tables and analysed by the Chi-squared test. To investigate whether a combination of parameters was more predictive for the nodal status than a single parameter, stepwise logistic regression analysis was performed using the variables that showed significant correlation with metastases in the univariate analysis.

Survival curves were calculated using the Kaplan–Meier product limit estimate [10]. Deaths from causes other than the index tumour or its metastases were not considered treatment failures, and these patients were censored in all analyses involving the length of survival. Differences between survival times were analysed by the log-rank method [11]. Multivariate Cox proportional hazards models [12] were used to examine the relative impact of either variables demonstrated to be statistically significant in the univariate analysis or those variables likely to have an effect on outcome (e.g. tumour size and histopathological grade). In these models, E-cadherin staining score was dichotomised as above a percentile 75 (score = 225) versus below this percentile. Similarly, T-stage classification was dichotomised as T1, T2 or T3 versus T4. Lymph node metastasis classification was dichotomised as N0 versus N1, N2 or N3. Finally, histopathological grade was dichotomised as well and moderately differentiated versus poorly differentiated. Dichotomisation was made based on a previous analysis of the survival in each category of the variable. For E-cadherin, survival curves of the staining scores among the different percentiles were obtained before dichotomisation. The cut-off points which provided better discrimination among the survival curves were chosen. P values of <0.05 were considered to be statistically significant.

3. Results

3.1. Patterns of expression in normal epithelium and primary carcinomas

E-cadherin showed membranous labelling in all the cells in the lower half of the normal squamous epithelium used as a positive control. Hence, only this half was considered to give the staining score of the normal mucosa surrounding the tumour. In all the 91 cases in which adjacent normal mucosa was stained, the normal appearing epithelium had a staining score of at least 200 (mean staining score of 368).

All but two carcinomas showed E-cadherin expression. Expression was generally weaker than in the normal epithelium; the mean staining score ($\pm$standard deviation) for the carcinomas was 136 ± 109 (median, 100). An example of staining for E-cadherin is shown in Fig. 1.

3.2. Association of E-cadherin expression with clinicopathological parameters

Table 2 presents the correlation of E-cadherin expression with T-stage, nodal metastases and pathological grading. The differences in expression between the different T stages were not statistically significant ($P = 0.09$). No relationship was observed between E-cadherin expression and histopathological differentiation ($P = 0.89$). There were significant differences in the mean staining scores of E-cadherin between those tumours with and without nodal metastases: lower staining scores were associated with nodal metastases ($P = 0.007$). Other parameters associated with lymph node metastases in the univariate analysis were increased T-stage ($P = 0.025$) and decreased histological differentiation ($P = 0.043$). The results of a stepwise logistic regression analysis showed that these three parameters (T-stage, histological grade and E-cadherin

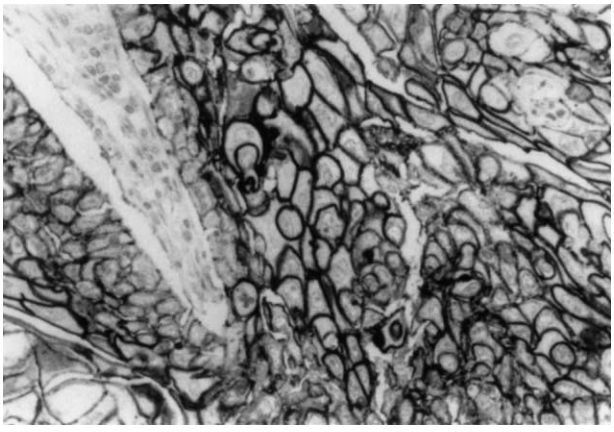

Fig. 1. An example of positive immunostaining for E-cadherin showing a membranous staining pattern (original magnification ×260).

Table 2
E-cadherin expression by clinico-pathological findings

Characteristic	Cases *n*	Mean E-cadherin expression	95% confidence interval for mean	*P* value[a]
Total cases	101	136	115–158	
pT stage				
T1	13	118	53–182	0.09
T2	38	171	132–210	
T3	37	110	79–141	
T4	13	128	62–193	
pN stage				
N0	46	168	133–202	0.007
N1-3	55	110	84–136	
Histological grade				
Well differentiated	45	144	112–177	0.89
Moderately differentiated	36	134	96–172	
Poorly differentiated	20	122	68–175	

[a] analysis of variance (ANOVA) test.

Table 3
Results of logistic regression model on lymph node metastases

Variable	Hazard Ratio	95% Confidence Interval	*P* value
T-stage	1.96	1.17–3.27	0.008
Histological grade	2.16	1.18–3.96	0.009
E-cadherin score	0.99	0.991–0.999	0.016

Table 4
Effect of recurrences on the staining scores in 80 patients with supraglottic squamous cell carcinoma

Recurrence	Cases *n*	Mean E-cadherin expression	95% Confidence Interval for mean	*P* value
No recurrence	47	154	126–182	0.019
Tumour recurrence:	33	99	68–131	
Local recurrence	8	125	36–213	
Regional recurrence	22	100	62–138	
Distant metastases	3	33	−67–133	

staining score) were statistically significant, independent predictors of nodal metastases (Table 3). Therefore, its combination results in a higher predictive value for nodal metastases. By combining T-stage, histological grade and E-cadherin score, an accuracy of 70% was achieved, with a sensitivity of 71%, and a specificity of 70%.

3.3. E-cadherin expression and the disease course

21 patients that died from causes not related to the index tumour were excluded from the recurrence analysis. During the follow-up period, 8 cases developed local recurrence, 22 regional recurrence and three distant metastases. The cases that presented tumour recurrence (grouping local recurrences, regional recurrences and distant metastases) had a mean staining score significantly lower than the cases without recurrence ($P=0.019$; Table 4). Interestingly, the lowest staining scores were found in the 3 cases that developed distant metastases (mean staining score of 33).

From the analysed parameters (E-cadherin expression, T-stage, N-stage and histological grade), the only one that had a statistically significant association with a reduced disease-specific survival was the presence of regional lymph node metastases (log-rank $P=0.0002$). There was a decline in 5-year survival associated with a decreased E-cadherin expression, but this was not significant (log-rank $P=0.061$). The results of multivariate Cox proportional hazards model confirmed that only the presence of cervical lymph node metastases was a statistically significant, independent predictor of a reduced disease-specific survival ($P=0.001$). Expression of E-cadherin did not enter in either of the multivariate Cox models ($P=0.16$). The univariate near statistical significant relationship of E-cadherin expression with survival is likely to be the result of its association with nodal metastases.

4. Discussion

An intact E-cadherin–catenin complex is required for maintenance of normal intercellular adhesion. In the light of this, several groups have proposed that in carcinomas E-cadherin functions as an invasion suppressor molecule such that its loss permits or enhances the invasion of adjacent normal tissues [1]. There is substantial data to support this hypothesis. *In vitro* studies have shown various human cancer cell lines with an epithelioid, differentiated morphology to be generally non-invasive and to express E-cadherin, whereas cell lines with a fibroblast-like morphology are invasive and

have often lost E-cadherin expression [13]. *In vivo*, immunohistochemical studies of different types of human cancers have frequently shown that a proportion of these neoplasms have reduced levels of E-cadherin expression in comparison to their related normal tissues (reviewed in Ref. [2]).

Abnormal expression of E-cadherin has been correlated in several human carcinomas with the pathological characteristics of the tumour, such as tumour stage, grade of differentiation, invasiveness, lymph node involvement and distant metastases [14–17]. Moreover, reduced expression of E-cadherin has been correlated with clinical variables, such as disease relapse and disease-free survival [18–20]. However, studies of E-cadherin expression in squamous cell carcinomas of the head and neck have failed to provide a clear picture for its role in these tumours.

Generally, E-cadherin expression was found to be high in well differentiated cancers, but reduced in undifferentiated cancers [3–8]. Our results show a general, but not significant, decline in E-cadherin expression with increasing dedifferentiation of the tumour. In relation to other clinicopathological variables, especially lymph node involvement and survival, previous studies have reported conflicting results. Some studies have demonstrated a correlation between reduced E-cadherin expression and nodal metastases [3–5], whereas others have failed to show this relationship [7–9]. Similarly, an association between E-cadherin expression and survival was suggested in some studies [6], whereas others did not found any correlation of E-cadherin expression with recurrences or survival [7,8]. Several explanations for this discrepancy can be suggested [2]. The site and number of cases analysed, selection of tumours (stage, tumour grade), differences in surgical approach (extent of lymph node dissection), and differences in staining evaluation may individually or in combination be responsible. To avoid most of these problems, in our study we have selected a large number of cases of the same *site*, well balanced by tumour stage and grade, treated in the same way, and with a complete follow-up. In this population, we found that, in addition to other known factors such as T-stage and differentiation status, E-cadherin expression was an independent predictor of lymph node metastases. This is consistent with another study on laryngeal tumours [5], and with large studies in other histological types [14–16]. In addition, we have found a significant association of E-cadherin expression with tumour recurrence. However, we failed to found any significant association of E-cadherin expression with survival, and the pathological N-stage was the only factor that was independently associated with survival. Since the presence of lymph node metastases in the histopathological examination is the most important prognostic factor in squamous cell carcinomas of the head and neck [21], the correlation of E-cadherin expression with tumour recurrence is probably due to its association with nodal metastases. Therefore, for squamous cell carcinomas of this site, it is clearly not a better prognostic indicator than histological nodal stage.

Nevertheless, the determination of E-cadherin expression in the primary tumour might be a powerful tool in clinical practice as a marker to predict the metastatic potential in individual patients with supraglottic squamous cell carcinomas and clinically negative lymph nodes. Of course, other factors like T-stage and histological grade (as we have demonstrated) must be taken into account in the decision to treat or not a N0 neck with a neck dissection. The combination of these parameters in our study resulted in a high correlation with and predictive value for nodal metastases, although not strong enough to guide clinical management. Since metastasis is a multistep process, it is not likely that a single marker will be able to predict metastatic behaviour of all of the tumours. Possibly a characteristic expression pattern of a set of invasion factors will be more predictive in determining the metastatic potential of a single tumour. It seems that when these factors can be identified it may be possible to evaluate the nodal status of patients more reliably by studying the biopsy material of the primary tumour, which is relatively easily accessible in most head and neck squamous cell carcinomas. Meanwhile, the immunohistochemical determination of E-cadherin expression (that seems to be one of these factors involved in the metastatic process) gives us an instrument to characterise the metastatic potential of these carcinomas. This determination can be performed easily on standard paraffin-fixed pathology specimens, facilitating its inclusion in the diagnostic work-up and treatment planning of these tumours. This is especially true in the treatment of the N0 neck. In these cases, E-cadherin expression may play a role in the decision to treat a N0 neck with a neck dissection or with close follow-up (in addition to other factors like T-stage, histological differentiation, age and co-morbidity).

It has to be noted that in this study only surgical specimens were analysed and a comparison with the original biopsies was not performed. It is possible that the expression analysis of a sample of another portion of the same tumour would show another result. The problem of tumour heterogeneity in determining the histological grade is well known to pathologists. To compare the results of an expression analysis with the clinicopathological parameters, we have to presuppose that the analysed samples were representative of the entire tumour.

In conclusion, the E-cadherin adhesion molecule is abnormally expressed in supraglottic squamous cell carcinomas. Reduction of E-cadherin expression in these carcinomas is an independent predictor of lymph node metastases, and their immunohistochemical determination might 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. However, E-cadherin expression does not appear to be a better indicator of patient outcome than established markers. Future prospective trials are required to confirm the role of E-cadherin expression in predicting the behaviour of these neoplasms.

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