Prognostic Significance of N-Cadherin Expression in Oral Squamous Cell Carcinoma

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Abstract. *Background: N-Cadherin (CDH2) is a calcium-dependent adhesion protein, whose de novo expression, re-expression, up-regulation and down-regulation in human tumors has been demonstrated. The aim of the present work was to define the prognostic role of N-Cadherin in a large series of oral squamous cell carcinomas (OSCCs). Materials and Methods: A total of 94 selected OSCCs were quantitatively and qualitatively analyzed by immunohistochemistry for N-Cadherin. The association between protein expression and clinico-pathological parameters was assessed by statistical analysis. Results: In neoplastic tissue, N-Cadherin levels were more evident than in normal peritumoral epithelium (p<0.05). Protein staining was mainly detected in the neoplastic cells, and only focal nuclear positivity was observed. Expression of cytoplasmic N-Cadherin correlated significantly with poor histological differentiation (p<0.05). Furthermore, we have observed significant a statistical trend for stage and a correlation with worst patient outcome, also confirmed by Kaplan-Meier estimates. Conclusion: Our work has underlined the key-role of N-Cadherin in oral carcinogenesis and in the prognostic stratification of patients.*

Oral squamous cell cancer (OSCC) represents one of the major health issues, with over 200.000 new cases reported annually, worldwide (1). Although improvements in screening and early diagnosis have dramatically reduced the incidence of this neoplasm in recent years, the 5-year disease-free survival is still poor, despite the great scientific and financial efforts. For many years, the main prognostic factors of (OSCC) have been the conventional grading, staging and site of the tumor. Nowadays, the goal of scientific research is to find new biological markers able to define the tumor's biological 'fingerprint' and to identify set(s) of genes involved in oral carcinogenesis and in cancer prognosis.

Cadherins are cell adhesion multifunctional proteins that have important roles in normal development, morphogenesis, organogenesis and carcinogenesis (2, 3). These proteins are surface molecules regulating homotypic calcium-dependent cell-cell adhesion and have been classified as classical cadherin, E-Cadherin, N-Cadherin, P-Cadherin, and non-

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classical cadherin, OB-Cadherin, VE-Cadherin, K-cadherin, LI-cadherin, BR-cadherin, M-cadherin, R-cadherin, T-cadherin (4). Although it is well documented, cadherins' role in establishing and maintaining epithelial stability by regulating cell adhesion, recent evidence suggests that cadherins play different and numerous important functions in various aspects of cell biology, including the control of cell polarization, differentiation, stemness and cell motility.

E-Cadherin is the predominant cadherin in simple epithelium, including glandular, squamous, and transitional types. N-Cadherin has been found in neural tissue (5), but it is also expressed in cells of mesenchymal and mesodermal origin. Cadherin's profiles may be used in diagnostic pathology. In neoplasia, tissue-specific patterns of cadherin expression have been useful in discriminating tumors of similar histology but with distinct subtypes or cell type origin.

Cadherins are well-known tumor suppressor genes. This concept has been best demonstrated for E-Cadherin (6). However, although other members of the cadherin family have been reported as tumor suppressors (7), their role in different types of cancer is very complex. N-Cadherin is expressed in mesothelial cells and the corresponding tumors express or overexpress N-Cadherin. In some epithelial tumors, P-Cadherin and N-Cadherin show aberrant expression, generally related to an aggressive biology and poor prognosis.

N-Cadherin (CDH2) is a calcium-dependent adhesion protein located on chromosome 18q11.2. Several studies from current literature have shown *de novo* expression, re-expression, up-regulation and down-regulation of N-Cadherin in human tumors and tumoral cell lines(8). In particular, breast, prostate, bladder and thyroid cancer have shown *de novo* N-Cadherin expression (9-11). Melanoma, gastric carcinoma, chordoma, rhabdomyosarcoma and (ATL) and T-cell leukemia demonstrated re-expression of N-Cadherin. Leiomyoma, pheochromocytoma, adrenocortical carcinoma and mesotheliomas showed N-Cadherin overexpression. Ovarian carcinoma, osteosarcoma, glioblastoma and renal oncocytoma demonstrated N-Cadherin down-regulation (10).

The aim of the present retrospective study was to evaluate N-Cadherin expression in a series of oral squamous cell carcinomas (OSCCs) and to assess its association with clinical and histopathological parameters.

Materials and Methods

Study population. Upon approval by the Ethical Committee of the all Institutions, we analyzed 94 OSCCs, selected from the Anatomic Pathology informatic Archive of the Torrette Regional Hospital – Ancona (1990-2007). Demographical and clinico-pathological characteristics of the study population are summarized in Table I. The survival rate was calculated from the date of surgery to the date of the latest clinical follow-up or death due to the disease or to other causes. Patients underwent oral surgery without previous treatment. All patients received surgical or radio/chemotherapic treatment only

Table I. *Baseline characteristics of the 94 patients with OSCC.*

Variable	Category	N (%)
Age (years)	<64	40 (42.5)
	≥64	54 (57.5)
Gender	Male	65 (69.1)
	Female	29 (30.9)
Stage	I	29 (30.8)
	II	25 (26.6)
	III	15 (16)
	IV	25 (26.6)
Differentiation	Well	31 (33)
	Moderate	45 (47.9)
	Poor	18 (19.1)

with curative intention. All patients or their relatives gave their informed written consent. The histopathological diagnosis, reporting the grade and stage of all OSCCs, was performed and carefully reviewed by two expert pathologists (BP/AS) at the University of Foggia, Department of Surgical Science, Section of Anatomic Pathology and Cytopathology. Tumour extent determined from clinical records, computed tomography and magnetic resonance imaging data, was revised and classified according to the 2002 TNM classification. Special care was taken in assessing tumour nuclear grade on paraffin-fixed, haematoxylin and eosin (H&E)-stained sections. Moreover, an adequate group of normal oral mucosa was identified for the comparative analyses.

Immunohistochemistry. Histological and immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue samples. Immunostaining was performed using the linked streptavidin-biotin horseradish peroxidase technique (LSAB-HRP). Antigen retrieval was performed by microwave heating, firstly time for 3 min at 650 W, then twice for 3 min at 350 W, the slides being immersed in 10 mM citrate buffer (pH 6). After heating, the sections were blocked for 60 min with 1.5% horse serum (Santa Cruz Biotechnology, CA, USA) diluted in PBS buffer before reaction with the primary antibody (Ab). The primary polyclonal anti-human N-Cadherin Ab [N-Cadherin N-19, sc-1502; Santa Cruz Biotechnology] was diluted 1:400 and sections were incubated overnight. After two washes with PBS, the slides were treated with biotinylated species-specific secondary antibodies and streptavidin-biotin enzyme reagent (DAKO, Glostrip, Denmark), and the color was developed by 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin and mounted using xylene-based mounting medium. In negative controls, the primary antibody was omitted. The results of the immunohistochemical staining were separately evaluated by two independent observers by examining at least 10 high-power fields (HPFs) with an optical microscope (OLYMPUS BX41, at ×40); for each case, the average percentage of positive cells in all sections examined was determined and scored in three categories: score 1 (0-20% of positive cells); score 2 (21-40% of protein expressing cells); score 3 (>40% of protein expressing cells). Inter-rate reliability between the two investigators blindly and independently examining the immunostained sections was assessed by Cohen's K-test, yielding K-values higher than 0.70 in almost all instances.

Table II. *ANOVA results. Statistical univariate analysis of N-Cadherin immunohistochemical expression and associated pathological findings of OSCCs.*

Variable	Category	Sc1 (n=25)	Sc2 (n=33)	Sc3 (n=36)	*p*-Value
Stage	I	12	8	9	0.018
	II	5	9	11	
	III	7	4	4	
	IV	1	12	12	
Differentiation	Well	13	9	9	0.043
	Moderately	8	18	19	
	Poor	4	6	8	

The statistical evaluation of immunohistochemical data has been compared to pathological reports. An overall higher degree of protein expression than normal peri-tumoral oral epithelium ($p<0.05$) has been found in OSCC. Significant correlations between N-Cadherin protein expression and histological degree and stage was observed. Sc: score of protein expression. Synthetically, the number of N-Cadherin expressing tumour cells was estimated as a percentage of the final number of cells per section and scored in three categories: score 1 (0-20% of positive cells); score 2 (21-40% of protein expressing cells); score 3 (>40% of protein expressing cells).

Staining patterns were also evaluated and recorded; staining was cytoplasmatic in all cases.

Western blotting. Total protein extracts from tissues were obtained using a lysis buffer contained NP40 0.5% 50 mM HEPES (1 M) (pH 7), 250 mM NaCl (5M) with phosphatase and protease inhibitors. Protein extracts from tissues (80 μg/ml) were separated by (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were stained with a 10% Ponceau S solution to verify equal loading and transfer efficiency before being blocked with 5% milk at room temperature for one hour. Then the membranes were incubated overnight with monoclonal antibodies to E-Cadherin (BD Transduction Laboratorie), N-Cadherin (BD Transduction Laboratories) and γ-tubulin (Santa Cruz Biotechnology). Finally, after incubation with secondary antibodies for one hour and washing throughly, the signal was detected with the aid of a chemiluminescence kit (ECL; Amersham Pharmacia Inc).

Statistical analysis. The data were analyzed by Stanton Glantz statistical software 3 (MS-DOS) and Graph-Pad Prism software version 4.00 for Windows (Graph Pad Software, San Diego, CA, USA; www.graphpad.com). Differences between groups were determined using the one-way analysis of variance (ANOVA) and the Student-Newman-Keuls test to evaluate the correlation between N-Cadherin expression and clinicopathological parameters (Table II). *P*-values <0.05 were regarded as significant. Kaplan-Meier curves were used for the survival analysis of the patients.

Results

Clinicopathological features. Ninety-four patients with OSCC were studied, encompassing 65 men and 29 women, with ages ranging from 29 years to 91 years (mean, 64

years). In 61 cases (64.9%) the tumour was localized in the mouth (floor, lip), and in 33 cases (35.1%) in the tongue. Thirty-one (33%) OSCCS were well-differentiated (G1), 45 (47.9%) were moderately differentiated (G2) and 18 (19.1%) poorly differentiated (G3). There were 29 (30.8%) stage I, 25 (26.6%) stage II, 15 (16%) stage III and 25 (26.6%) stage IV tumours (AJCC/UICC classification).

Immunohistochemical analysis in different types of OSCC. By immunohistochemistry, we firstly assessed the different expression levels of N-Cadherin in dysplastic and neoplastic oral epithelium. The proliferating cells in the dysplastic basal layer had strong cytoplasmic expression (Figure 1), while a different subcellular pattern of expression was noted in carcinomatous cells.

An overall up-regulation was found in neoplastic cases with respect to normal cells (Figure 2). In particular, a case exhibiting a droplet invasion pattern revealed strong N-Cadherin cytoplasmic-nuclear expression, and another case with single-cell pattern of invasion exhibited intense membranous-cytoplasmic N-Cadherin staining. We also compared N-Cadherin expression with E-cadherin staining in the same tumoral cases that we have analyzed in another study on OSCC (data not shown). In particular, a case of OSCC with a cord-type pattern of invasion exhibited co-expression of E-cadherin and N-Cadherin in deeply invading cells (Figure 3). In fact, we generally noted E-cadherin membranous expression in the superficial layer of OSCC and its suppression in deep invasion, together with an absence of N-Cadherin staining in superficial well-differentiated cancer cells and strong staining in deeply infiltrating cancer cells (Figure 4). Finally, we found a statistically significant correlation between N-Cadherin expression and some histopathological parameters, including grade (1 *vs*. 2 and 3 $p=0.018$), and a statistical trend for stage (1 to 4; $p=0.04$), as shown in Figure 5.

Expression of N-Cadherin in OSCC tissues. In order to evaluate N-Cadherin expression in OSCC specimens, total proteins were extracted from one representative case of oral squamous cell carcinoma and from normal tissue used as control. As shown in Figure 6, the OSCC sample exhibited overexpression of N-Cadherin protein, whereas the expression level was lower than that of the control.

Survival analysis. Survival data were available in 48 cases, with a mean follow-up of 49.02 months. By Kaplan-Meier curves, survival estimates showed that OSCCs overexpressing N-Cadherin (cut-off >61%) are characterized by a worse prognosis, in terms of overall survival (*p*-value not significant, but a statistical trend was noted) and disease-free survival, with a more evident tendency for relapse and metastasis (*p*-value not significant, but a statistical trend was noted) (Figure 7).
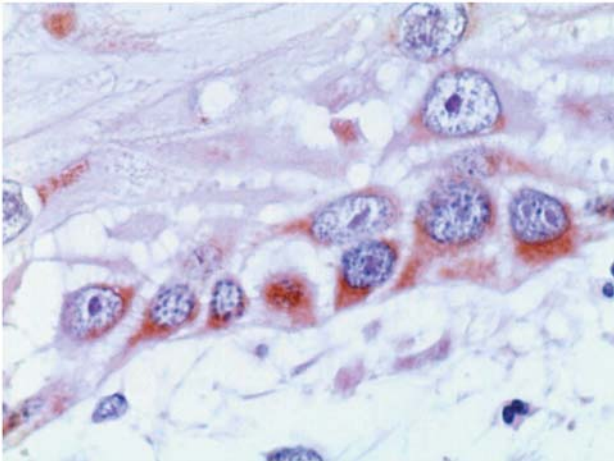
N- CAD

Figure 1. *Immunohistochemical expression of N-Cadherin in dysplastic oral epithelium. Strong expression in the basal layer of proliferating cells. LSAB-HRP, nuclear counterstaining with Mayer's hematoxylin, original magnification ×400.*

Discussion

The term epithelial-to-mesenchymal transition (EMT) is defined as the switching of polarized epithelial cells to a migratory fibroblastoid phenotype, describing a process in which epithelial cells lose their characteristic polarity, disassemble cell-cell junctions and become more migratory (12). EMT is proposed to occur in various developmental processes and during tumor metastasis. The idea of EMT, and its role in development and/or tumour progression, is still controversial, but it is well known that beta-catenin and the so-called 'cadherin switch' can have a profound effect on cell phenotype and behavior (13).

The loss of E-Cadherin expression or its functional elimination represents a key step in the acquisition of the invasive phenotype for many tumour types. Recent evidence indicates, however, that in addition to the loss of the 'invasion-suppressor' E-Cadherin, another adhesion molecule, N-Cadherin, becomes up-regulated in invasive tumour cell lines. N-Cadherin was shown to be present in the most invasive and de-differentiated breast cancer cell lines, and its exogenous expression in tumour cells induces a scattered morphology and heightened motility, invasion, and metastasis. N-Cadherin probably also supports the systemic dissemination of tumor cells by enabling circulating tumor cells to associate with the stroma and the endothelium at distant sites. Some authors have declared that the majority of the cadherin/catenin complex can be formed prior to cleavage of the E-cadherin pro-region. The catenins, including α-catenin, β-catenin, and p120ctn, bind to immature cadherin while it is travelling
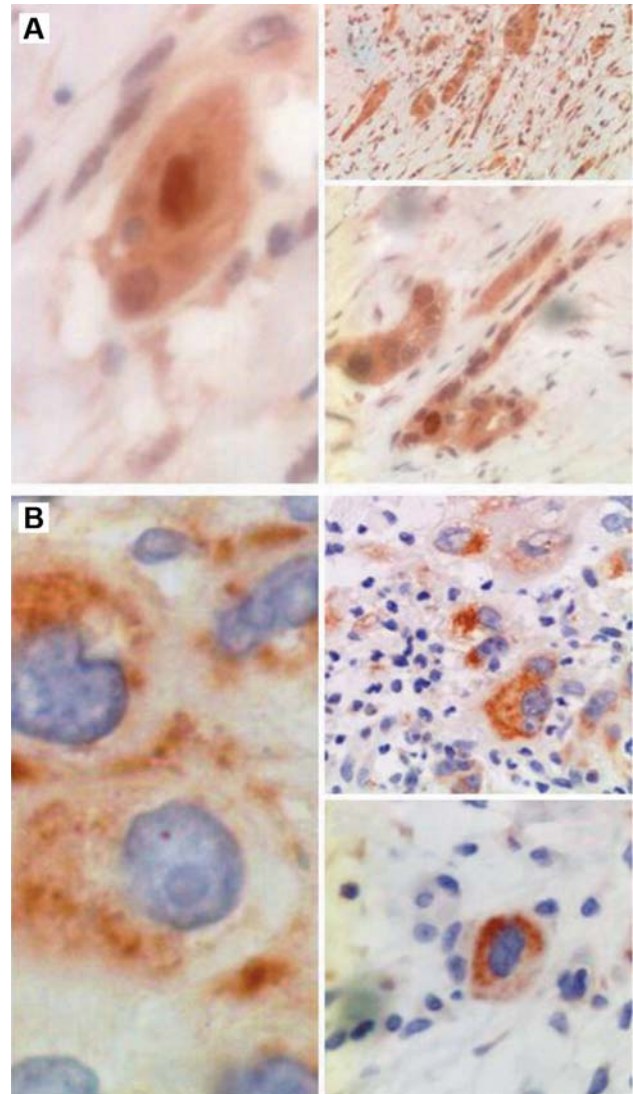

Figure 2. *N-Cadherin up-regulation in two representative cases of OSCC. An OSCC case showing droplet invasion pattern and strong N-Cadherin cytoplasmic-nuclear expression (A). Another OSCCC case with single cell pattern of invasion with intense membranous-cytoplasmic N-Cadherin staining (B). LSAB-HRP, nuclear counterstaining with hematoxylin.*

through the endoplasmic reticulum and Golgi apparatus (14). It is well known that N-Cadherin interacts with beta-Catenin. This interaction was modeled on a demonstrated interaction between mouse N-Cadherin and beta-catenin (15, 16). Thus, although E-cadherin and N-Cadherin promote very different cellular phenotypes and behaviors, in a same cellular context, the cadherin/catenin complexes form in the very similar manner. It is likely that the interactions of cadherins with other proteins at the cell surface, such as growth factor receptors, are responsible for the distinct cellular phenotypes observed when a cell expresses different cadherins.
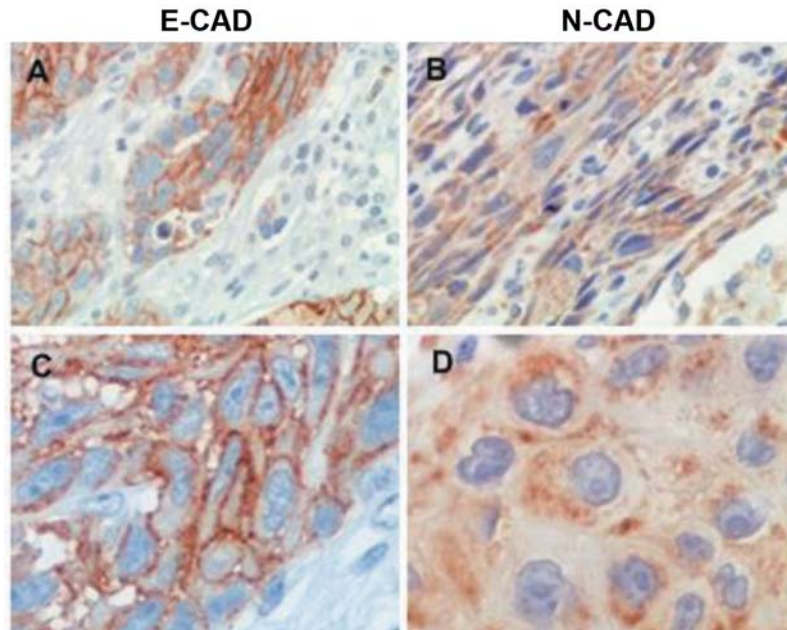
E-CAD   N-CAD

Figure 3. *Immunohistochemical expression of N-Cadherin and E-Cadherin. A case of OSCC with cord-type pattern of invasion showing co-expression of E-Cadherin (A, C) and N-Cadherin (B, D) in deeply invading cells. LSAB-HRP, nuclear counterstaining with hematoxylin. Original magnification A, B ×200; C, D, ×400.*
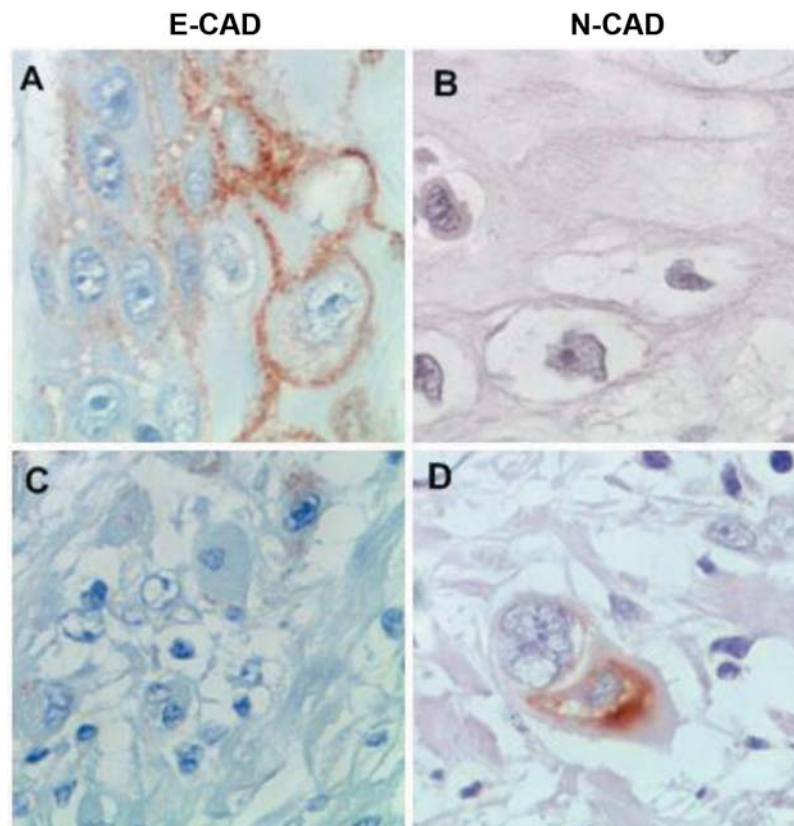
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Figure 4. *Cadherin switching in deep invasion from a representative case of OSCC. Membranous expression of E-cadherin in superficial layer of OSCC (A) and its suppression in deep invasion (C) together with lack of staining for N-Cadherin in superficial well-differentiated cancer cells (B) and strong immunostaining in deeply infiltrating (single-cell pattern) cancer cells (D). Serial section immunostaining with LSAB-HRP technique, nuclear counterstaining with hematoxylin, original magnifications ×400.*
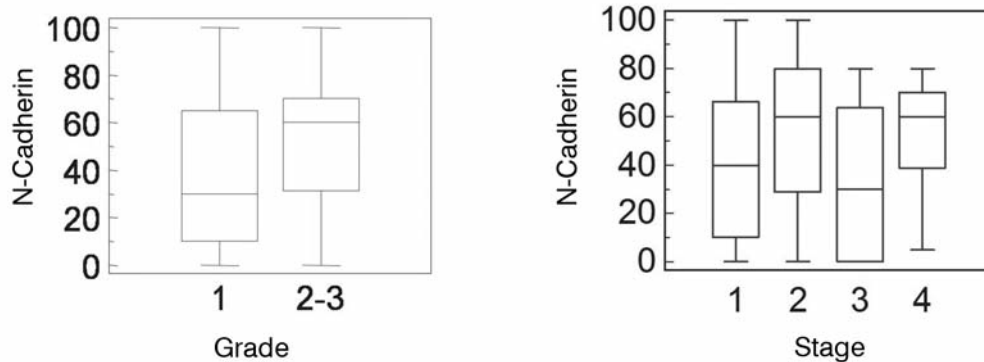
Figure 5. *N-Cadherin expression according to grade and stage. There was a statistically significant correlation between N-Cadherin expression and grade (1 vs. 2 and 3, p=0,018), and a statistical trend for stage (I-IV, p=0.04).*

Aberrant N-Cadherin expression and E-Cadherin/N-Cadherin switching (EN-Switch) involved in EMT represent an independent prognostic marker in cancer progression; this concept has been well documented in gastric, prostate and oral carcinomas (17-19). Furthermore, some studies demonstrate that cadherin switching is necessary for increased motility but is not required for the morphological changes that accompany EMT (20), therefore, immunohistochemical detection should be performed in order to detect the EN-switch and the consequent EMT in oral cancer.

Current literature on the prognostic role of N-Cadherin has been limited and characterized by short follow-up and lack of complete and detailed information about diagnosis and therapy. To our knowledge, the present study could be considereds a large retrospective analysis to investigate the association between N-Cadherin expression in OSCC and the clinicopathological characteristics of the lesions. Our work has focused on the prognostic role of the cytoplasmic and also focally nuclear N-Cadherin expression. To our knowledge, the literature does not report studies on nuclear N-Cadherin expression in oral cancer cells. Some authors demostrated that Merkel cell carcinomas showed marked preference for nuclear versus membrane localization of E-Cadherin, whereas small cell tumors from other sites showed fewer cases of nuclear molecular expression. They also concluded that the nuclear localization of E-Cadherin did not correlate with cadherin-associated protein beta-catenin nuclear expression (21).

In our OSCCs, the nuclear pattern of N-Cadherin expression was particularly observed in dedifferentiated cancer, characterized by a worse prognosis. Therefore, we maintain that the pattern of cadherin expression might constitute a useful diagnostic and prognostic tool in the evaluation of tumors and for determining the histogenesis of tumour cells. Moreover, we found a statistically significant correlation between N-Cadherin expression and grade (1 *vs.* 2 and 3, $p=0.018$), and a statistical trend for stage (1 to 4; $p=0.04$). Kaplan-Meier curves also showed that OSCCs
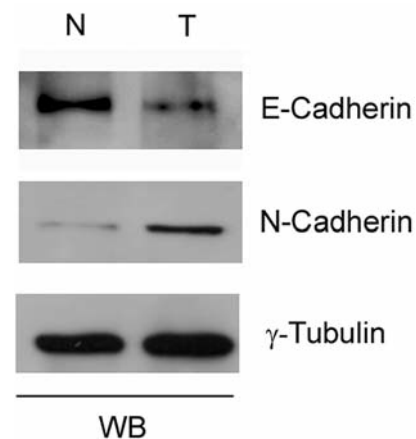

Figure 6. *Expression of E-Cadherin and N-Cadherin in a representative case of oral squamous cell carcinoma tissue. Western blot analysis using the indicated antibodies in whole cellular extracts from a representative case of oral squamous cell carcinoma tissue with respect to normal tissue. γ-Tubulin was used as loading control.*

overexpressing N-Cadherin (cut-off >61%) are characterized by a worse prognosis and a major tendency for relapse and/or metastasis.

Finally, because the search for molecular prognostic markers for oral cancer is still a major clinical and therapeutic issue, our data have highlighted the importance of detecting the expression of N-Cadherin in clinical practice and in the diagnostic protocol of OSCC.

Conclusion

Squamous cell carcinoma is the most frequent malignant tumour of the oral cavity. The five-year survival rate for patients with oral and oropharyngeal cancer in developed countries is still poor (40-50%), comparable to the five-year survival rate in the 1970s, despite advances in detection,
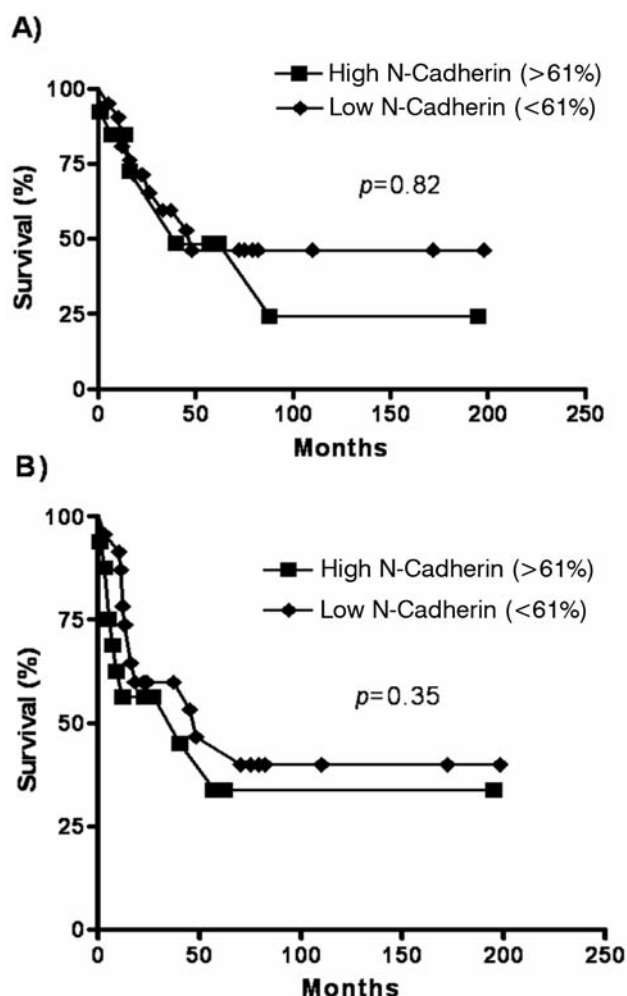
Figure 7. *Kaplan-Meier estimates. Survival estimates have shown that OSCCs overexpressing N-Cadherin (cut-off >61%) are characterized by a worse prognosis, in terms of overall survival (A: no significant p, but a statistical curve trend is notable) and disease free survival, with a more evident tendency to relapse and metastasis (B: no significant p, but a statistical curve trend is notable).*

surgery, radiation and chemotherapy. Thus a preventive approach before the development of invasive cancer is highly desireable and novel strategies to reduce cancer incidence are being pursued. The molecular mechanisms involved in oral carcinogenesis are still not yet well understood, nor has the complete genetic profile of cancer cells been characterized. Since an abnormal, unregulated, cellular adhesion mechanism is a peculiar feature in neoplastic diseases, cellular interactions represent a crucial point in cancer development and progression. In our work, univariate and survival analyses showed that OSCCs with a high percentage of N-Cadherin expression were characterized by a malignant phenotype associated with a worse clinical outcome, reduced overall survival and a major tendency for loco-regional invasion and

metastasis. This study underlines the central role of N-Cadherin in oral carcinogenesis, underlining its function as a potential therapeutic target and its potential value as a prognostic tool in the clinical management of OSCC.

Acknowledgements

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