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P-cadherin expression predicts clinical outcome in oral squamous cell carcinomas

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Summary. P-cadherin, a transmembrane molecule similar to E-cadherin involved in the cell-cell adhesion, and catenins form complexes between its cytoplasmic domain and the cytoskeleton. Five cell lines, 108 specimens of oral squamous cell carcinomas (OSCC), 9 metastasis and 10 of normal oral mucosa were examined to evaluate P-cadherin expression and cellular localization by immunohistochemistry and western-blotting. In normal oral mucosa there was a membranous expression only in basal and parabasal layers. 91 cases (84%) showed membranous/cytoplasmic positivity, whereas 17 cases (16%) were negative. In particular, while well-differentiated carcinomas showed P-cadherin upregulation, the protein was homogeneously hypo- or unexpressed in low-differentiated carcinomas. There was a statistically significant correlation between P-cadherin expression and tumour grading: G3 tumours had a lower score than G1-G2 tumours ($P<0.05$). When analysed for prognostic significance, patients with no P-cadherin expression (score 0) had poorer overall and diseases-free survival rates than the P-cadherin-expressing group (score 1) ($P=0.0463$ and $P=0.0471$, respectively). Western blotting analysis of cell lines and tissue samples confirmed immunohistochemical findings. When cell staining pattern of positive cases was examined, 52 cases showed a prevalent membranous pattern, while 39 had a prevalent cytoplasmic pattern. Cases with prevalent cytoplasmic staining showed high rates of lymph node metastases ($P>0.05$), and regional relapse ($P <0.05$) and poorer survival rates than the group with prevalent membranous expression ($P<0.0001$). An absent P-cadherin expression could constitute a hallmark of aggressive biological behaviour in oral squamous cell carcinoma.

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Introduction

Invasive head-neck cancers, in spite of improved therapeutic procedures, actually show a generally poor prognosis for metastases. As is well-known, the incidence of lymph node metastases is significantly correlated with the clinical stage and the localization of primary tumours (Berenson et al., 1989), as well as with the differentiation of tumoral cells and their skill for adherence (Shear et al., 1976; Yamamoto et al., 1984; Frierson et al., 1986; Umeda et al., 1992).

Intercellular adhesiveness is mediated by a family of glycoproteins named cadherins (Takeichi et al., 1988), which are composed of an extra-cellular domain, involved in Ca^{++}-dependent homophilic binding to adjacent cells, a trans-membrane domain, and a intra-cellular domain which binds to proteins called catenins (Gumbiner and McCrea, 1993). In epithelial cells, this adhesiveness is mediated by epithelial-cadherin (E-cadherin), a 120-kd transmenbrane glycoprotein, localized mainly in the zonula adherens junctions. The cadherin family includes other members: neural-cadherin (N-cadherin) (Hatta and Takeichi, 1986), placental cadherin (P-cadherin) (Nose and Takeichi., 1986) and liver cell adhesion molecule (L-CAM), and more than 20 cadherins have been described in the central nervous system, liver and vascular endothelial cells and in other tissues and organs (Suzuki et al., 1991; Buxton et al., 1993).

P-cadherin is a protein homologous to E-cadherin: it is a calcium-dependent cell-cell adhesion molecule which mediates homophilic and homotypic adhesion between cells in contact (Takeichi, 1991). Both

molecules are preferentially concentrated in the adherens type of intercellular junctions, where these molecules interact with cytoskeleton by alpha-catenin. Both cadherins are cell-adhesion molecules expressed in solid tissue (Takeichi, 1991) and are important for initiating and maintaining cell-cell contact and cell polarity (McNeill et al., 1990). Binding of E-cadherin to alpha-catenin and, hence, the formation of the zonula adherens, is mediated by beta- or gamma-catenin, which act as adaptors between cadherins and alpha-catenin. P-cadherin also shows similar rapport with catenin, but P-cadherin is detected on the cell-cell contact surface of basal keratinocytes in normal mouse and human epidermis. Naturally, cells migrating into the suprabasal compartiment down-regulate P-cadherin expression.

P-cadherin is expressed in mouse placenta (Nose and Takeichi, 1986), epithelia, including lung epithelia (Hirai et al., 1989a), the basal cell of the skin (Hirai et al., 1989b; Shimoyama et al., 1989), myoepithelial cells of the mammary gland (Daniel et al., 1995), and the outer root sheath and hair matrix of the hair follicle (Nose et al., 1986; Shimoyama et al., 1989; Fujita et al., 1992), and plays an important role in the morphogenesis of epidermis and skin appendage (Hirai et al., 1989b; Wheelock and Jensen, 1992; Lewis et al., 1994). The expression of P-cadherin in epithelial tissues appears to identify cell populations with proliferative activity, and its expression decreases as cells differentiate (Shimoyama et al., 1989; Hodivala and Watt, 1994).

The possible role exerted by the cadherin in human carcinogenesis has been suggested by a number of studies (for a review see ref. (Birchmeier and Behrens, 1994) and (Birchmeier, 1995)). Down-regulation of E-cadherin was reported to be directly related to invasiveness and progression of many human epithelial tumours (Birchmeier, 1995), including oral squamous cell carcinomas (OSCC) (Downer and Speight, 1993).

While E-cadherin expression has been extensively studied in many forms of human cancers, including OSCC (Schipper et al., 1991; Bowie et al., 1993; Mattijssen et al., 1993; Sakaki et al., 1993, 1994; Birchmeier and Behrens, 1994; Kinsella et al., 1994; Sakaki et al., 1994; Schipper et al., 1994; Fuller et al., 1996; Andrews et al., 1997; Bagutti et al., 1998; Williams et al., 1998), less is known about the expression levels of P-cadherin in human cancers (Shimoyama et al., 1989; Shimoyama and Hirohashi, 1991; Rasbridge et al., 1993; Yasui et al., 1993; Palacios et al., 1995; Pizarro et al., 1995; Shimoyama et al., 1995; Foty and Steinberg, 1997; Matsuyoshi et al., 1997; Paul et al., 1997; Soler et al., 1997; Sanders et al., 1998) and to date there are only three recent studies on its expression in OSCCs in vivo (Sakaki et al., 1994; Bagutti et al., 1998; Williams et al., 1998). The final goal of this study was to evaluate the role of this protein in the carcinogenetic process of the oral cavity.

Therefore, we have retrospectively evaluated P-cadherin expression in 108 human OSCCs with a different degree of cellular differentiation and 5 cell lines.

Materials and methods

Cell lines

5 cell lines were used: KB and KM4 (poorly-differentiated OSCC cell line), KM2 (well-differentiated OSCC cell lines) and KM3 and KM5 (moderately-differentiated OSCC cell line). KM2, KM3, KM4, and KM5 cell lines were kindly sent by Prof. Masaki Okafuji, Department of Pathology, Yamaguchi University School of Medicine, Ube, Japan. Cell lines were cultured as monolayers in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mmol L-glutamine, 400 U/ml penicillin and 200 µg/ml streptomycin and kept at 37 °C in a humidified atmosphere with 5% CO_2 in air.

Selection of cases

108 samples from paraffin embedded specimens of primary oral squamous cell carcinomas with different degrees of cellular differentiation, 9 from paraffin embedded specimens of lymph node (7 cases) and tissutal (2 cases) metastases of OSCCs included in this study were used. Paraffined specimens were fixed in 10% neutral-buffered formalin. 20 frozen specimens of human OSCCs included in this study were used for western blotting.

None of the patients had previously been treated. They received surgical treatment with a curative intention. No single case of this study concerned patients with contemporary multicentric lesions. Cases in this study were included only when a complete clinical follow-up, ranging from 12 to 96 months was available. Clinical data were reviewed to record sex and age of patient, and site and size of the lesion. The group consisted of 77 men and 31 women with a mean age of 67.05 years (range 20-88). The histopathological grading was assessed on paraffin H&E-stained sections. Tumour extent was classified according to the TNM system by UICC(UICC, 1987) and tumours were divided into grades 1, 2 and 3 using the WHO histological classification (Washi et al., 1971).

Ten paraffined and five frozen specimens of healthy oral mucosa were obtained from patients who had undergone routine oral surgical procedures (such as impacted third molars, metaprotesic reactive epithelial hyperplasia….) with the informed consent of the donors. The use of archived human tissues conformed to an informed consent protocol that had been reviewed and approved by the institutional review board.

Western blotting

Frozen tissue and cellular pellets were homogenized into protein extraction reagent (T-PER Tissue, Pierce, USA) containing protease inhibitor cocktail (Sigma-

Aldrich, St. Louis, Missouri, USA) and clarified by centrifugation at 14,000 x 15 min at 4 °C. Protein concentrations were estimated using the Bradford assay (Sigma-Aldrich, St. Louis, Missouri, USA).

Subsequently, 20 µg of total protein extracts were heated to 95°C for 5 min and electrophoresed on 10% polyacrylamide gel (SDS-PAGE) under reducing conditions. Proteins were transferred to a nitrocellulose membrane (Bio-Rad, Melville, NY, USA) overnight at 90 mM and 4 °C; complete transfer was assessed using prestained protein standards (BenchMark Pre-stained, Invitrogen). Blots were placed in PBS, pH 7.5, containing 5% non-fat dried milk and 0.1% Tween-20 (blocking solution) and incubated for 60 min at 37 °C. After washing in PBS containing 0.1% Tween-20, blots were incubated with available mouse monoclonal IgG antibody against P-cadherin (Transduction Laboratories, Lexington, Kentucky, USA), packaged at 0.25 mg/ml, and used at a dilution of 1:250 in blocking solution for 60 min at room temperature (RT). The membranes were then washed five times with PBS containing 0.1% Tween-20 before incubation with the secondary antibody, anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) 1:10000 in blocking solution for 60 min at RT. Blots were finally washed five times in PBS containing 0.1% Tween 20, processed with Super Signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) and autoradiographed. To confirm equal protein loading per lane, membranes were subsequently reacted with a 1:5000 dilution of a mouse monoclonal antibody to ß-actin (Sigma-Aldrich, St. Louis, Missouri, USA)

Immunohistochemistry

5 µm serial sections from routinely formalin-fixed paraffin-embedded blocks were cut for each case, and one section stained with haematoxylin-eosin (H.E.) was used to confirm the histopathological diagnosis. Only sections showing sufficient epithelium to assess 1000 cells were considered for this study.

Immunocytochemistry was then performed on the remaining sections mounted on poly-L-lysine-coated glass slides. Endogenous peroxidase was quenched by incubating the sections for 20 minutes with 0,3% hydrogen peroxide in methanol. To improve the staining pattern, the sections were boiled three times for 3 minutes in 10 mM citrate buffer as an antigen retrieval method. In order to prevent non-specific binding of antibodies, sections were then pre-incubated with non-immune mouse serum (1:20; Dakopatts, Hamburg, Germany) and diluted in PBS/BSA (1%) for 25 minutes at room temperature. After washing twice with Tris-HCl buffer, primary antibodies were applied. As positive controls, the immunoreactivity of normal skin sections was evaluated. A negative control was also performed in each run by substituting primary antibodies with non-immune serum (DAKO Antibody Diluent, Dakopatts, Hamburg, Germany). All the slides were washed twice in Tris-HCl buffer between each step.

Commercially available mouse monoclonal IgG antibody against P-Cadherin (Transduction Laboratories, Lexington, Kentucky, USA), packaged at 0.25 mg/ml, was used at a dilution of 1:300. The specificity of this antibody has been described in the literature (Soler et al., 1997).

Evaluation of immunostaining

The number of P-cadherin-expressing tumour cells was estimated as a percentage of the final number of 500 neoplastic cells of each case, and scored in two categories: score 0 (< 5% of cells were positive), score 1 (P-cadherin expression in > 5 of cells %). The expansion of P-cadherin-positive cells in the spinous layer was defined as anomalous P-cadherin expression (Pizarro et al., 1995). The topographical staining pattern (membranous, cytoplasmic, membranous/cytoplasmic) was examined separately and scored in two categories: prevalent membranous (>50% of cells showed membranous pattern) or prevalent cytoplasmic (>50% of cells showed cytoplasmic pattern).

Statistical analysis

Statistical analysis was carried out with the use of one-way analysis of variance and the Student-Newman-Keuls test. These tests were performed using the Stanton A. Glantz statistical software version 3.0 (McGraw-Hill, Milan, 1994). The probabilities of overall survival and progression-free survival were calculated using Kaplan-Meier estimates. Survival probability distributions were compared with the log-rank test (Mantel-Cox). Crude and adjusted hazard ratios were calculated using Cox's proportional hazard regression analysis. All Cox models appeared to appropriately fit the data. Statistical significance was established at the 0.05 alfa-value and accordingly 95% confidence intervals (CI) around hazard ratios are presented. All P values were derived from two-sided tests.

Results

P-cadherin expression in oral cell lines

Expression levels of P-cadherin in oral cell lines were assayed by western blotting (Fig. 1a). Anti-P-cadherin Mab recognized bands ranging from 90 to ~115 kDa; the protein was detectable in some cell lines. An expression was observed in KM2, KM3, KM4, and KM5, while KB, a poorly-differentiated cell line, showed no expression of the protein. The intensity of the ß-actin protein was almost equal in all cell samples. These last results suggested a correlation between the P-cadherin expression pattern and the degree of cellular differentiation, but they regarded expression levels only, without any distinction of intracellular localization of the protein.

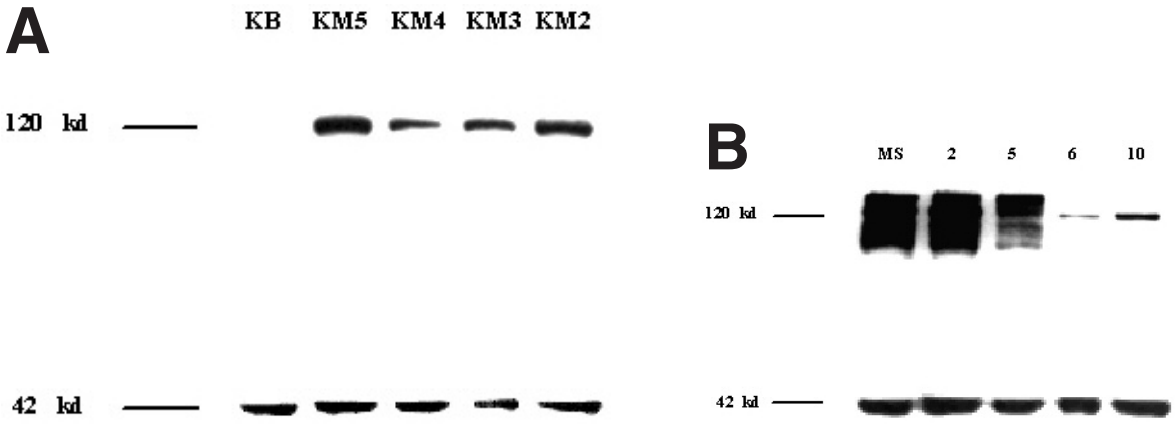

Fig. 1. Western blotting showing expression levels of P-cadherin (~110 Kda) in protein extracts from cell lines **(panel A)** and tissue samples **(panel B)**. P-cadherin is expressed by KM5, KM4, KM3, KM2 cell lines. KB is negative. There is a correlation between differentiation degree (KB and KM4: poorly differentiated; KM3 and KM5: moderately differentiated; KM2: well differentiated) of cancer cell lines and expression pattern of P-cadherin **(A)**. Oral cancer expresses different levels of P-cadherin with respect to normal mucosa. A reduced expression of P-cadherin corresponds to poorly-differentiated carcinomas (n. 6, 10). Well-differentiated carcinomas (n. 2, 5) show no reduction in expression of protein towards normal mucosa (MS) **(B).** In both panels expression of ß-actin (42 Kda) is simultaneously tested as internal control.

Table 1. Statistical analysis of P-cadherin expression and associated clinicopathological findings in OSCCs.

VARIABLES	No.	SCORE 0 n. (%)	SCORE 1 n. (%)	MEAN	STANDARD DEVIATION	STANDARD ERROR	P < 0.05	STATISTICAL DATA			
Cases	108	17 (16)	91 (74)								
Age											
< 65 years	50	7	43	0.840	0.370	0.052					
> 65 years	58	10	48	0.844	0.365	0.047	No°	P = 0.945			
Sex											
Male	77	15	52	0.805	0.398	0.045					
Female	31	2	29	0.935	0.249	0.044	No°	P=0.094			
Grading									Mean		
G1	35	2	33	0.942	0.235	0.039			Diff.	q	P value
G2	52	7	45	0.865	0.344	0.047	Yes*	grade 3 vs grade 1	-0,323	4,734	< 0.01
G3	21	8	13	0.619	0.497	0.108		grade 3 vs grade 2	-0,246	3,845	< 0.01
								grade 2 vs grade 1	-0,077	1,430	> 0.05
Size											
< 1.5 cm	33	6	27	0.818	0.391	0.068					
> 1.5 cm	75	11	64	0.853	0.356	0.041	No°	P = 0.6477			
Lymph node metastasis											
Negative	77	11	66	0.857	0.352	0.040					
Positive	31	6	25	0.806	0.401	0.072	No°	P = 0,5174			
Staging									Mean		
I	53	9	44	0.830	0.379	0.052			Diff	q	P value
II	20	2	18	0.900	0.307	0.068	No*	stage 4 vs stage 2	-0,100	1,210	>0.05
III	15	2	13	0.866	0.351	0.090		stage 4 vs stage 3	-0,066	---	>0.05
IV	20	4	16	0.800	0.410	0.091		stage 4 vs stage 1	-0,030	---	>0.05
								stage 1 vs stage 2	-0,069	---	>0.05
								stage 1 vs stage 3	-0,036	---	>0.05
								stage 3 vs stage 2	-0,033	---	>0.05
Relapse											
Yes	25	6	19	0.760	0.4359	0.037					
No	84	11	72	0.867	0.3411	0.087	No°	P = 0,1993			

°: Student-Newmann-Keuls test. *: One-way Analysis of Variance (ANOVA) and Student-Newman-Keuls Multiple Comparisons Test.

P-cadherin expression in normal mucosa.

Squamous cells in the basal layers of skin sections showed strong membranous staining (Moles and Watt, 1997). All 10 normal oral specimens showed intense homogenous membranous P-cadherin staining (Fig. 2a,b), as observed in the epidermis (Moles et al., 1997), predominantly on the membrane of only a thin line of basally located cells, with occasionally moderate parabasal staining (Bagutti et al., 1998). Membranous staining was observed at the basal layer of histologically normal oral epithelium present in the OSCC specimens. The intensity of staining for P-cadherin progressively reduced from basal to parabasal layers and stopped in the spinous layer. No staining for P-cadherin was observed in the upper layer (Fig. 2a,b).

P-cadherin expression in OSCC

Areas of dysplastic transformation showed membranous and/or cytoplasmic P-cadherin up-regulation. Well-differentiated oral carcinomas showed

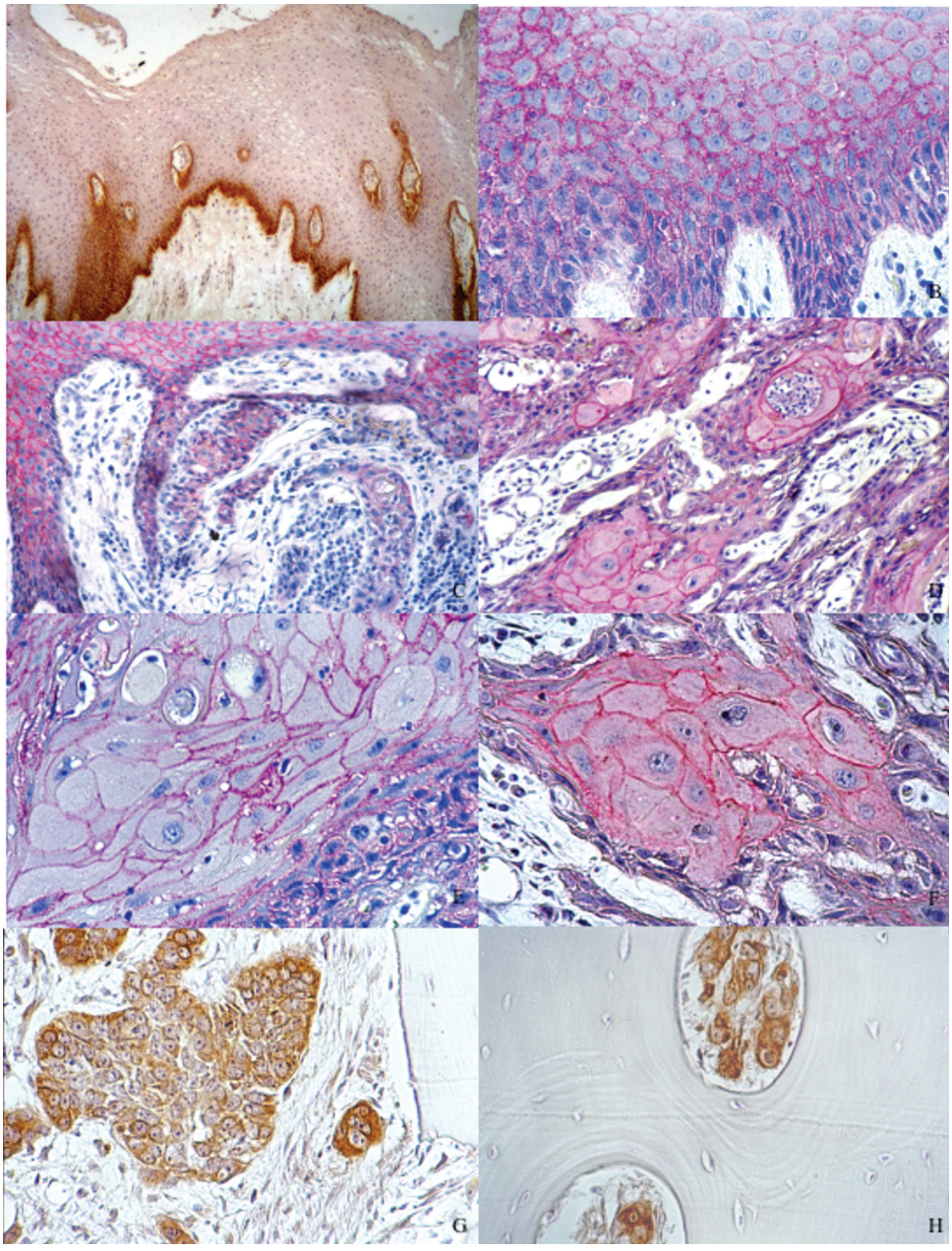

Fig. 2. a. Strong basal-parabasal expression of P-cadherin in oral hyperplastic epithelium (LSAB-HRP, nuclear counterstaining with haematoxylin, x 106). **b.** P-cadherin expression in hyperplastic area of the oral mucosa (LSAB-AP, x 250). **c.** Membranous P-cadherin expression in oral moderately-differentiated SCC. Note positivity in rete ridge of the lower lip epithelium and progressive loss of the membranous staining pattern in areas of microinfiltration. (LASB-AP, x 250) **d.** Membranous P-cadherin expression in oral well-differentiated SCC (LASB-AP, x 250) **e.** Higher magnification: membranous P-cadherin expression in oral well-differentiated SCC. (LASB-AP, x 400). **f.** Membranous expression of P-cadherin in an area of stromal infiltration from well-differentiated OSCC (LASB-AP, nuclear counterstaining with haematoxilin, x 400) **g.** Cytoplamic expression of P-cadherin in a case of MD-SCC infiltrating stroma near bone (LSAB-peroxidase, x 250). **h.** High expression of P-cadherin in a case of bone metastasis from MD-SCC (LSAB-peroxidase, x 400).

P-cadherin up-regulation (Fig. 2c-f), while P-cadherin expression homogeneously reduced in less differentiated oral squamous cell carcinomas (grade 3). 17 cases of carcinoma (16%) showed no positivity and 91 cases (84%) showed membranous/cytoplasmic positivity (Fig. 2g). In positive cases the pattern was not always homogeneous with membranous expression on peripheral cells in the tumor islands. In poorly-differentiated carcinomas expression of p-cadherin shifted to membranous/cytoplasmic co-localization, predominantly cytoplasmic in distribution, or alternatively was absent in a large numbers of cells.

Definite P-cadherin positivity was present in 6 out of 7 (85%) lymph node metastases and in 2 out 2 (100%) bone metastases (Fig. 2h). In lymph node metastases, P-cadherin positivity was observed in both the primary site and the metastatic foci in the lymph nodes in 7 out of 7 cases (100%). The staining pattern was always cytoplasmic (Fig. 2h).

There was no statistically significant correlation between P-cadherin expression and sex, recurrence, size, staging or lymph node metastases. There was a statistically significant correlation between P-cadherin expression and grading: G3 tumours had a lower score than G1-G2 tumours ($p<0.05$).

Survival curves were performed in relation to stage (Fig. 3A), grade (Fig. 3B), lymph node (Fig. 3C) and size (Fig. 3D) of tumours. When analysed for prognostic significance, patients with negative P-cadherin expression (score 0) had poorer overall survival rates than the group with positive expression (score 1) (Fig. 4A). This difference of survival rates was statistically significant (P=0.0463). Also when analysed for disease-free survival, patients with negative P-cadherin expression (score 0) had poorer survival rates than the group with positive expression (score 1) (Fig. 4B). This difference of survival rates was statistically significant (P=0.0471).

When the cell staining pattern of positive cases was examined, 52 cases showed a prevalent membranous pattern, while 39 had a prevalent cytoplasmic pattern (Table 2). Cases with prevalent cytoplasmic staining showed a high rates of lymph node metastases, even if they were not statistically significant (P>0.05), and of regional relapses which were statistically significant (P=0.0001) (Table 2).

When analysed for prognostic significance, patients with positive P-cadherin expression (score 1) had different survival rates in relation to cell pattern: patients with prevalent cytoplasmic staining had poorer survival

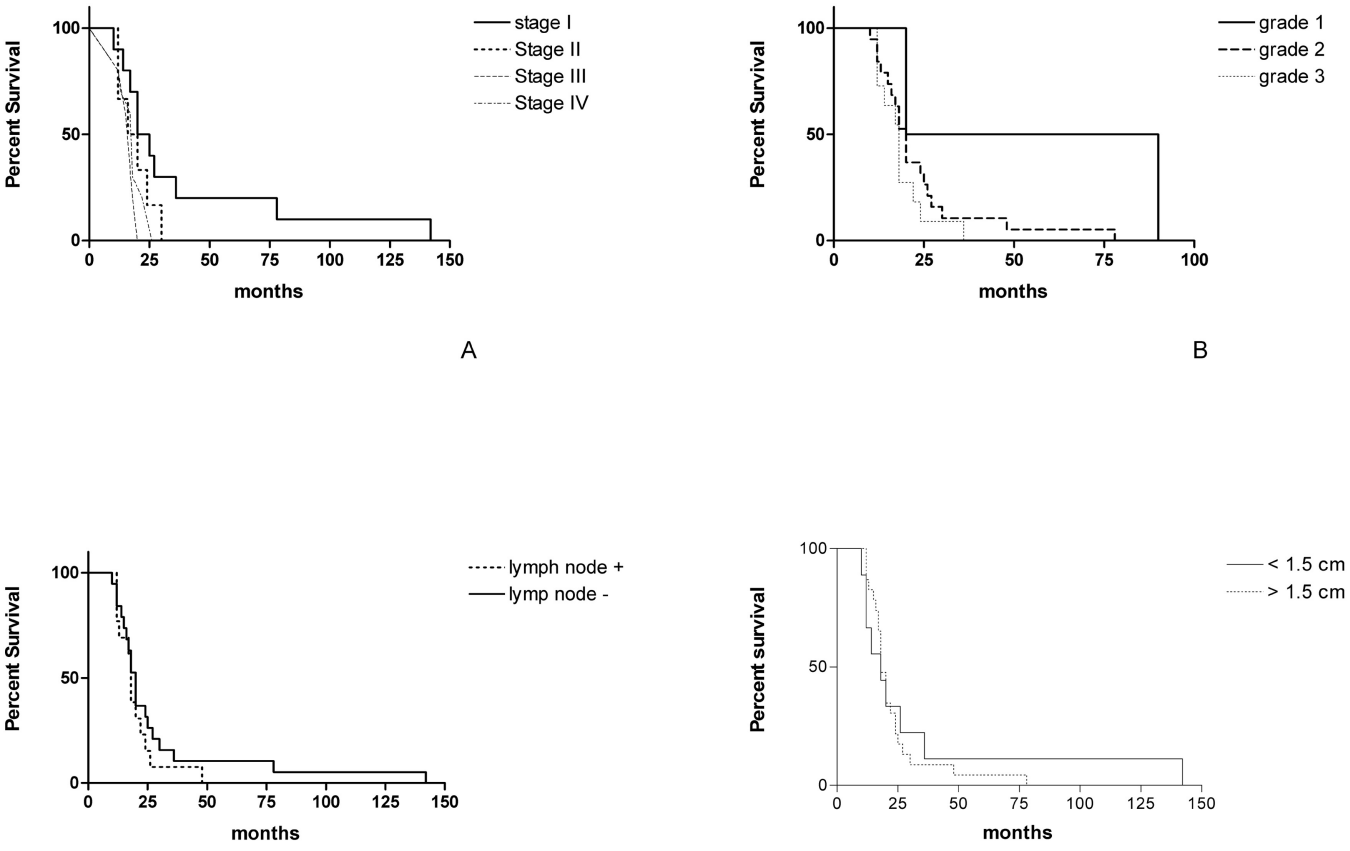

Fig. 3. Survival curves related to stage **(A)**, grade **(B),** lymph node **(C)** and size **(D)**.

rates than the group with prevalent membranous expression (Fig. 4C). This difference of survival rates was statistically significant (P<0.0001).

In conclusion, patients had different survival rates in relation to staining pattern: patients with no staining had poorer survival rates than the group with prevalent cytoplasmic and prevalent membranous expression (Fig. 4D). This difference of survival rates was statistically

Table 2. Statistical analysis of P-cadherin cell expression (membranous or cytoplasmic) and associated clinicopathological findings in OSCCs.

VARIABLES	No.	MEAN	STANDARD DEVIATION	STANDARD ERROR	P<0.05	STATISTICAL DATA
Cases	91					
Lymph node metastasis						
Membranous	11/52	0.2115	0.4124	0.057		
Cytoplasmic	14/39	0.3589	0.4860	0.077	No°	P = 0,1216
Relapse						
Membranous	4/52	0.076	0.2691	0.037		
Cytoplasmic	15/39	0.384	0.4929	0.078	yes°	P = 0,0003

°: Student-Newmann-Keuls test

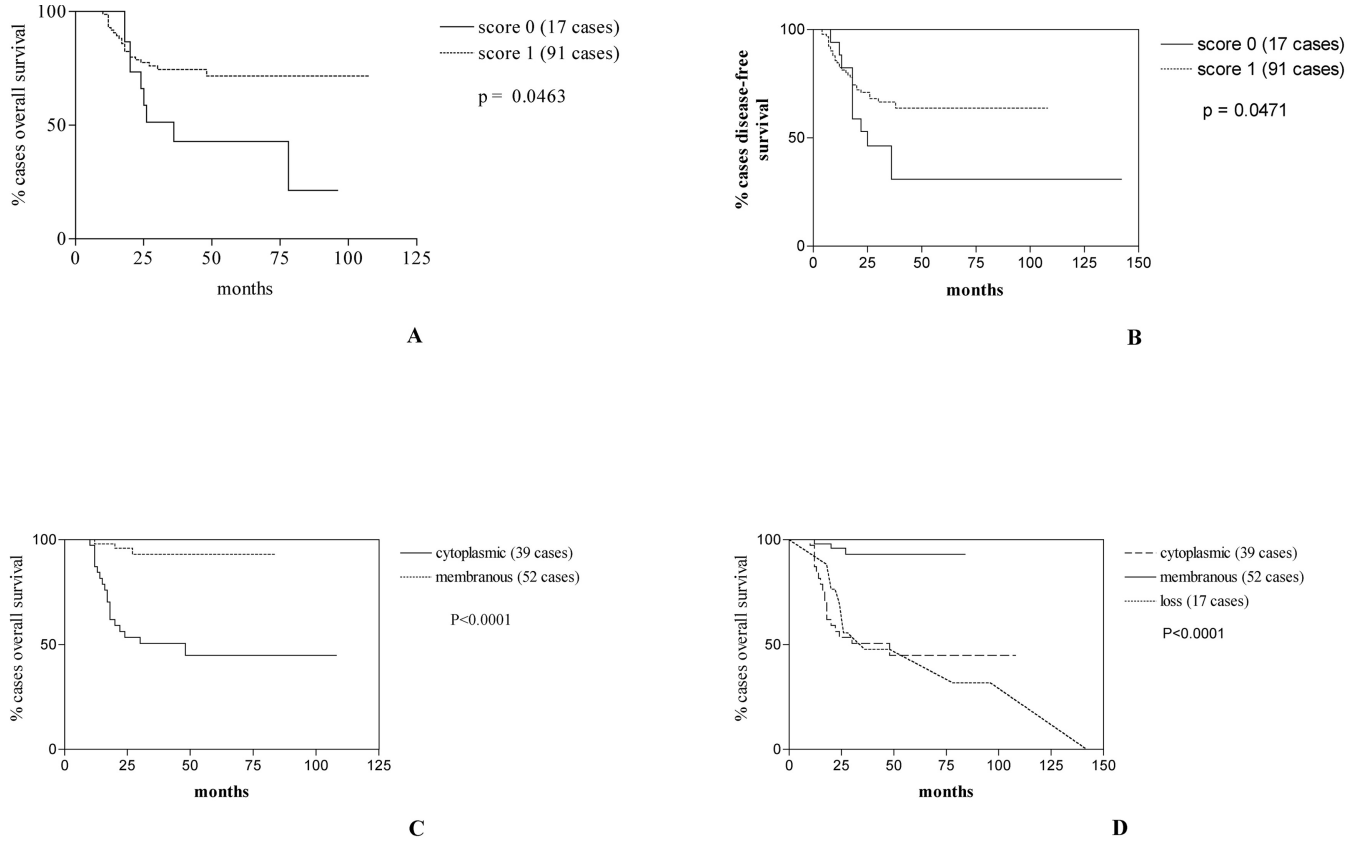

Fig. 4. A. Overall survival curves. Patients with negative P-cadherin expression (score 0) have poorer survival rates than the group with positive expression (score 1). This difference of survival rates is statistically significant (P<0.05). **B.** Disease-free curves. Patients with negative P-cadherin expression (score 0) have poorer rates than the group with positive expression (score 1). This difference of survival rates is statistically significant (P<0.05). **C.** Overall survival curves. Patients with positive P-cadherin expression (score 1) have different survival rates in relation to cell pattern: patients with prevalent cytoplasmic staining have poorer survival rates than the group with prevalent membranous expression. This difference of survival rates is statistically significant (P<0.0001). **D.** Overall survival curves. Patients have different survival rates in relation to staining pattern; patients without staining have poorer survival rates than the group with prevalent cytoplasmic and prevalent membranous expression. This difference of survival rates is statistically significant (P<0.0001).

significant (P<0.0001).

In addition to P-cadherin status, tumour stage and grade were significantly associated with survival in the univariate analysis. In the multivariate analysis, P-cadherin expression was a significant independent predictor of survival when simultaneously assessed with age, sex, size, grade, and stage (95% CI, 0.0561 to 0.5032) (P<0.0001).

Expression of P-cadherin in tissue extracts of normal mucosa and SCC was also demonstrated by western blotting (Fig. 4B). P-cadherin was detectable in all normal mucosa and some OSCCs but at different levels. Well-differentiated carcinomas showed nearly or exactly the same protein expression when compared to normal mucosa, while the reduced expression was notable in poorly-differentiated carcinomas. All samples had a comparable expression of ß-actin.

Discusion

The E- and P-cadherin play a pivotal role in the maintenance of the epithelial structure, even if they are expressed in distinct regions of the epithelium: E-cadherin is expressed on all epithelial layers, while P-cadherin is predominantly expressed in the basal layer of stratified squamous epithelia, the proliferative compartment (Nicholson et al., 1991; Fujita et al., 1992; Takeichi, 1995; Borradori and Sonnenberg, 1996; Shirahama et al., 1996; Moles et al., 1997).

E- and P-cadherin expression is altered in premalignant and malignant skin tumors, as demonstrated by reduced E-cadherin and aberrant P-cadherin expression in human squamous cell carcinomas (Shirahama et al., 1996), indicating the importance of coordinated cadherin expression for maintaining normal epidermal structure (Wakita et al., 1997).

In skin SCC, these cadherins showed a deregulation of their expression, even if with different grade; E-cadherin expression is reduced, whereas P-cadherin expression is generally increased, suggesting a relationship between E-cadherin reduction and the invasive/metastatic features of carcinoma and between aberrant P-cadherin expression and the proliferative cancer cells (Wakita et al., 1997). Malignant keratinocytes probably acquire different mechanisms for regulating the expression of these two cadherins (Wakita et al., 1998).

Our data showed a P-cadherin expression in basal and parabasal layers of normal oral mucosa. These cells represent the proliferative compartment of the epithelium. In the intermediate and superficial layers, where the cells lose ability to proliferate during maturation and stratification, there was a complete loss of P-cadherin expression. Areas of dysplastic transformation showed membranous and/or cytoplasmic P-cadherin up-regulation in agreement with other studies on oral mucosa (Williams et al., 1998), cervical mucosa (de Boer et al., 1999) or gastrointestinal mucosa (Sanders et al., 1998, 2000).

Well-differentiated oral carcinomas showed normal P-cadherin expression or up-regulation, while P-cadherin expression was homogeneously reduced in low-differentiated oral squamous cell carcinomas (grade 3) or its localization shifted to the cytoplasm, in accordance with other studies on oral mucosa (Williams et al., 1998) or gastrointestinal mucosa (Sanders et al., 1998, 2000). Williams et al. (1988) reported a loss of membranous immunostaining at the periphery of the islands of carcinoma with a cytoplasmic immunostaining or a complete loss. In contrast, towards the centre of the islands the more differentiated cells showed mild or moderate membranous staining in well- or moderately-differentiated carcinomas, reflecting the pattern seen in dysplasia (Williams et al., 1998). Also, in SCC of the skin P-cadherin was generally preserved, especially in cancer pearls (Wakita et al., 1998). P-cadherin membranous immunostaining in carcinomas probably mimicks the upregulation of dysplasias, while the cytoplasmic relocalization or loss may reflect a new or a loss of function of P-cadherin.

P-cadherin seems to play a role in the maintenance of the epithelial phenotype and may be involved, together with E-cadherin, in the final stage of tumor progression in epidermal carcinogenesis, being a marker of hyperproliferative activity (Pizarro et al., 1995). Studies on epidermis (Hodivala and Watt, 1994), gastric epithelium (Shimoyama et al., 1991), and mammary epithelium (Daniel et al., 1995) showed that P-cadherin controls cell proliferation in these tissues.

P-cadherin expression seems to be related to tumour progression in gastric (Yasui et al., 1993) and gingival carcinomas (Sakaki et al., 1994), while its expression is higher in poorly differentiated than in well-differentiated lung carcinomas (Shimoyama et al., 1989). However, a study on oesophageal squamous carcinoma demonstrated an increased expression of P-cadherin early in tumour genesis with loss of cadherin-catenin complexes in poorly-differentiated invasive carcinomas (Sanders et al., 1998). Probably in oesophagus, as in skin and oral mucosa, co-expression of E- and P-cadherin in the basal cell compartment may partly explain the maintenance of an undifferentiated phenotype in the proliferative compartment of basal cells (mutual inhibition) (Sanders et al., 1998).

Our data showed no positivity in 17 cases of carcinoma (16%) and 8 of these were G3. In cases with positivity the pattern was not always homogeneous with membranous expression on peripheral cells in the tumour islands. In poorly-differentiated carcinomas expression of P-cadherin shifted to membranous/cytoplasmic co-localisation, predominantly cytoplasmic in distribution, or alternatively was absent from a large numbers of cells. This pattern had already been described for beta- and gamma-catenin in oral squamous cell carcinoma (Lo Muzio et al., 1999). A recent study supported the existence of an inverse relationship

between the expression of both beta- and gamma-catenins and the degree of cellular differentiation and reported that the membranous expression of both catenins was homogeneously reduced in less differentiated oral squamous cell carcinomas (Lo Muzio et al., 1999). More interestingly, a decreased expression of these molecules was also found at the invasive front of moderate and sometimes of well-differentiated carcinomas, thus suggesting a more aggressive biological behavior of these cancer cells (Lo Muzio et al., 1999). The absence of catenin immunostaining in poorly-differentiated tumor cells can be explained by a loss of cell function and structure. In fact, the maintenance of adult tissue architecture is largely dependent on the function of cadherins (Bailey et al., 1998). The adhesive function of cadherins relies on the interactions with catenins. Many reports revealed reduced expression of cadherins and catenins in tumors, for example, in the progression of Barrett's esophagus to adenocarcinoma (Bailey et al., 1998), and in some specimens, catenin immunoreactivity was absent. Because of functional and architectural relations between catenin and cadherin, similar conclusions can also be drawn for P-cadherin.

Another study examined the immunohistochemical expression of cadherins and catenins during the process of oral carcinogenesis by comparing their expression in normal and dysplastic epithelium with primary and metastatic carcinomas (Williams et al., 1998). In oral SCC and in carcinoma in situ adjacent to infiltrating carcinomas, membranous expression of the cadherins and catenins was reduced or lost. In primary carcinomas, reduced membranous and cytoplasmic stainings were observed for both cadherins and catenins. Another study also showed reduced staining for the catenins in oral SCC (Bagutti et al., 1998), while the disruption of the E-cadherin/catenin complex is a late event associated with invasion (Williams et al., 1998).

There was no statistically significant correlation between P-cadherin expression and sex, relapse, size, staging or lymph node metastases. There was a statistically significant correlation between P-cadherin expression and grading; G3 tumours had a lower score than G1-G2 tumours ($p<0.05$). When analysed for prognostic significance, patients with negative P-cadherin expression (score 0) had poorer overall and disease-free survival rates than the group with positive expression (score 1). This difference in survival rates was statistically significant ($P<0.05$).

These data seem to show that P-cadherin expression does not prevent local invasiveness and aggressive behaviour of OSCC. In fact, cell-cell interactions mediated by P-cadherin seem to be more unstable than those mediated by E-cadherin (Wu et al., 1993); this phenomenon can probably be explained with the specific role of P-cadherin in cell-cell adhesion. P-cadherin is expressed only in the basal proliferating cells of stratified epithelia, where these cell-cell contacts are frequently broken and reformed (Nose and Takeichi, 1986; Shimoyama et al., 1989).

Anomalous P-cadherin expression in the spinous layer of epithelium overlying tumour can be a biological marker for keratinocyte atypia and/or premalignant changes. In fact, the continued expression of P-cadherin in the invasive cells can contribute to the maintenance of the epithelioid phenotype of the carcinoma cells (Cano et al., 1996). An experimental study on squamous cell carcinoma cell lines showed aberrant expression in cancer cells, whereas E-cadherin expression was reduced (Wakita et al., 1998). SCC cells probably acquire the ability to express P-cadherin and this molecule plays a role in tumour progression (Wakita et al., 1998). Elevated [Ca^{++}] determined increased cell-surface P-cadherin expression in SCC cell lines by up-regulation of de novo P-cadherin synthesis, while in normal keratinocytes calcium-induced cell-surface P-cadherin expression is a result of the translocation of pre-formed P-cadherin from the cytosol without up-regulation of P-cadherin synthesis (Wakita et al., 1997). These results suggest the existence of a unique mechanism for regulating the P-cadherin expression gene in tumour cells.

Bagutti et al. (1998) showed no correlation between P-cadherin expression and differentiation of tumour cells, while Sakaki et al. (1994) showed a complete loss of P-cadherin expression in poorly-differentiated gingival SCC.

There is probably a strict correlation between beta-catenin molecule and P-cadherin function. Tyrosine phosphorylation of beta-catenin inhibits the function of P-cadherin in v-src-transformed rat 3Y1 cells, although P-cadherin expression is not changed, showing a control of P-cadherin function by beta-catenin (Matsuyoshi et al., 1992).

In conclusion, the up-regulation of P-cadherin reflects an increase in the number of cells undergoing proliferation, as shown by its elevated expression in moderate and severe dysplasia of oral epithelium (Williams et al., 1998), while loss of P-cadherin expression is a late event prior to invasion, as shown by loss of its expression in dysplasia adjacent to infiltrating carcinomas (Williams et al., 1998). The loss of P-cadherin expression probably comes after P-cadherin cytoplasmic relocalization. Loss of P-cadherin expression in OSCC is associated with tumour invasion, while P-cadherin membranous staining in OSCC is probably due to the up-regulation seen in tumour cell lines and dysplasias. Therefore, in the initial phase of tumour growth the high expression of P-cadherin may be crucial in the formation of a tumor mass which is ready to progress and metastasize (Yasui et al., 1993). Then, cytoplasmic relocalisation or loss of P-cadherin expression may be responsible, together with other known/unknown upregulated oncogenes and downregulated tumour suppressor genes, for the later stages of tumor progression, such as invasive growth and

metastasis (Yasui et al., 1993).

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