Is expression of p120ctn in oral squamous cell carcinomas a prognostic factor?

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Objectives. p120ctn is a component of the catenin family. To date, there have only been two studies examining expression levels of p120ctn in oral squamous cell carcinoma (OSCC).
Materials and methods. Paraffined specimens of 113 OSCCs and 12 of normal mucosa were examined by immunohistochemistry. Frozen samples of 20 OSCCs and 5 of normal mucosa were examined by Western blot (WB). Results were correlated with clinicopathological parameters. Five cell lines were examined by immunofluorescence, immunocytochemistry, and WB to show immunoreactivity and cellular localization of p120ctn.
Results. Altered p120ctn expression was observed in 109/113 cases of OSCC. Heterogenous cytoplasmic/nuclear expression was associated with loss of membranous distribution (88/113 cases). Complete loss of expression was noted in 21/113 cases. Increased cytoplasmic expression was evident in all positive cases, without significant correlation among p120ctn staining/pattern and grading/stage. Reduction/absence of p120ctn expression was related to poor prognosis ($P < .05$).
Conclusion. p120ctn delocalization/loss of expression could be an independent prognostic marker in OSCC. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115:789-798)

The incidence of lymph node metastases is significantly correlated with the clinical stage and the localization of primary tumors, as well as with the differentiation of tumor cells and their adherence capability.[1,2] Intercellular adhesiveness depends on the glycoprotein family of cadherins.[3] These proteins present an extra-cellular domain that binds to adjacent cells, a transmembrane domain, and an intracellular domain which binds catenin proteins.[4] E-cadherin, a 120-kd transmenbrane glycoprotein, mediates epithelial adhesiveness function in the zonula adherens junctions.

Recently, a novel member of the catenin family, homologous to β- and γ-catenin, called p120ctn protein, has been described.[5] The human p120ctn gene (CTNND) is localized on the long arm of chromosome 11, in band 11q11.[6] p120ctn, β- and γ-catenin bind different regions of E-cadherin. In fact, p120ctn binds at the juxtamembrane region of the cytoplasmic domain, whereas β- and γ-catenin bind more distally.[7,8] For this reason, p120ctn coexists in E-cadherin complexes with either β- or γ-catenin.[7] It is thought that p120ctn may modulate adhesiveness of cadherins, but its exact role is unknown.[9] The possible role of the cadherin/catenin complex in human carcinogenesis has been suggested by several studies.[10,11] It is thought that down-regulation of E-cadherin is directly related to invasiveness and progression of many human epithelial tumor types,[11] including oral squamous cell carcinomas (OSCC).[12]

Moreover, it was also suggested that, given the role exerted by catenins for proper functioning of E-cadherin, loss of expression or mutations of these proteins might be functionally equivalent to E-cadherin loss of function.

Statement of Clinical Relevance

To the best of our knowledge, no studies have found a statistically significant correlation between p120ctn expression in OSCCs and patient survival, whereas the result of the present study clearly displayed that p120ctn delocalization/loss of expression seems to be a useful independent prognostic marker in oral squamous cell carcinoma.

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While E-cadherin and β-catenin expression have been extensively studied in many forms of human cancers, including OSCC,[13-16] less is known about the expression levels of p120ctn in human cancers[17-20] to date there have been no studies on its expression in OSCCs, except for our previous study involving on cell lines[21] and a very recent one that looked at potentially malignant oral lesions.[22]

Therefore, in the present study, we used immunohistochemistry to retrospectively evaluate p120ctn expression in 113 paraffined specimens of OSCC and 12 paraffined specimens of human oral normal epithelium. Moreover, 5 OSCC cell lines, 20 frozen specimens of human OSCCs, and 5 frozen specimens of human oral normal epithelium were investigated by immunocytochemistry, immunofluorescence, and western blotting (WB) techniques to evaluate p120ctn expression.

MATERIALS AND METHODS

Cell lines

Five cell lines were used: NCTC2544 (normal immortalized keratinocytes), OSC20 (well differentiated SCC cell line), CAL33 and CAL27 (moderately and poorly differentiated OSCC cell lines respectively), and KM5 (moderately differentiated gingival squamous cell carcinoma [SCC]). The NCTC-2544 cell line originated from skin keratinocytes of human origin (HL97002; Interlab Cell Line Collection, Genova, Italy).[23] KM5 cell line originated from a human oral squamous cell carcinoma of the tongue[24] and was kindly sent by Prof. Masaki Okafuji, Department of Pathology, Yamaguchi University School of Medicine, Ube, Japan. CAL27 and CAL33 cell lines originated from two human oral squamous cell carcinomas of the tongue[25,26] and were kindly sent by Prof. J. L. Fischel, Center Antoine-Lacassagne, Nice, France; CAL27 and CAL33 have relatively long doubling times (35 and 43 h respectively). OSC20 cell line originated from a human oral squamous cell carcinoma[27] and was kindly sent by Prof. N. Tanaka and Dr. H. Kondo, Department of Oral Surgery, Sapporo Medical University, School of Medicine, Sapporo, Japan.

NCTC2544, CAL33, and CAL27, and KM5 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, 200 μg/mL streptomycin and kept at 37°C in humidified atmosphere with 5% CO_2 in air. OSC20 cell line was cultured as monolayers in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mmol L-glutamine, 400 U/mL penicillin, 200 μg/mL streptomycin and kept at 37°C in humidified atmosphere with 5% CO_2 in air.

Oral specimens

113 primary OSCC samples, taken from surgical resection specimens, were included in this study. None of the patients had been treated previously. They received surgical treatment with curative intention. No case in this study involved patients with contemporaneous multicentric lesions. Clinical data were reviewed to record sex and age of the patient, and site and size of the lesion. Cases in this study were included only when a follow-up lasting at least 36 months was available. Patients who died of postoperative complications within 30 days were excluded. The group consisted of 77 men and 36 women with a mean age of 64 years (range 18-87). The histopathological grading was assessed on paraffin-embedded hematoxylin-eosin (H.E.)-stained sections, using the World Health Organization classification of histological differentiation (G1, G2, and G3), and tumor extent was classified according to the Tumor Node Metastasis (TNM) system by UICC (International Union Against Cancer, 2000). Twenty of these cases were also available as frozen specimens. Twelve paraffined and 5 frozen specimens of healthy oral mucosa were obtained from patients who had undergone routine oral surgical procedures with informed consent of the donors. Fresh oral tissue was obtained at surgery. Tumor material and normal mucosa were collected, snap frozen in liquid nitrogen, and stored at −80°C prior to use.

Immunocytochemistry

Cells were cultured on glass slides precoated with poly-L-lysine to enhance cell attachment. Cultured cells were rinsed with phosphate-buffered saline (PBS), fixed in cold acetone and air-dried. To improve the staining pattern, the slides were boiled 3 times for 3 min in 10 mM citrate buffer as antigen retrieval method. In order to prevent non-specific binding, sections were then preincubated with non-immune bovine serum (diluted 1:100 in PBS) for 25 min at room temperature (RT). After washing twice with Tris−HCl buffer, primary antibodies were applied. A negative control was performed in each run by substituting primary antibodies with non-immune reagent (Dako Antibody Diluent, Dakopatts, Hamburg, Germany). All the slides were washed twice in Tris−HCl buffer between each step. Primary antibody was diluted using 0.05 M Tris−HCl buffer pH 7.2-7.6, containing 1% bovine serum albumin and incubated at optimal dilution and time: mouse monoclonal IgG antibody against p120ctn (Transduction Laboratories, Lexington, KY, USA), packaged at 0.25 mg/mL, was used at a dilution of 1:500. Sequential 20-min incubations with biotinylated anti-mouse immunoglobulins and streptavidin conjugated to alkaline phosphatase were performed. Finally, a new fast red substrate system (K0597; Dako, Glostrup, Denmark) was applied as a chromogenic solution.

The number of p120ctn expressing tumor cells was estimated as a percentage of the final number of 300 neoplastic cells of each case, and scored in four categories: membranous (>90% of the cell showed a membranous positivity), cytoplasmic (>10% of the cell showed a cytoplasmic positivity), nuclear (the cell showed a nuclear positivity), and no staining.

Immunofluorescence microscopy

Cells were grown on glass coverslips, fixed with 4% paraformaldehyde in PBS for 15 min at RT, permeabilized with 0.5% Tryton-100 in PBS for 5 min, washed in PBS and blocked for 30 min in PBS containing 1% bovine serum albumin (BSA). Cells were incubated with p120ctn monoclonal antibody (Transduction Laboratories) at 1:500 dilution for 45 min at 37°C in a moist chamber, washed in PBS and stained with goat anti-mouse fluorescein-conjugated IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:200 dilution for 45 min at 37°C in a moist chamber. Finally, the coverslips were washed in PBS, mounted onto glass slides with 90% glycerol in PBS and examined with a Leica DMLB fluorescence microscope.[28]

WB

An aliquot of frozen tissue (30-50 mg) and cellular pellets were suspended in lysis buffer (PBS, containing 1% Nonidet P40, 0.1% sodium dodecyl sulphate (SDS), 1 mM phenylmethylsulfonylfluoride, 2 μg/mL aprotinin) and homogenized on ice using an Ultra-Turrax homogenizer (IKA, Staufen, Germany) at medium speed. The homogenate was centrifuged at 14,000 × 15 min at 4°C. Protein concentrations were estimated using Bradford assay (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as the standard.

Subsequently 50 μg of total protein extracts were heated to 95°C for 5 min and electrophoresed on 10% polyacrylamide gel (sodium dodecyl sulphate-polyacrylamide electrophoresis [SDS-PAGE]) under reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membrane (Bio-Rad, Melville, NY, USA); complete transfer was assessed using prestained protein standards (Bio-Rad). Blots were placed in PBS, pH 7, 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 p120ctn monoclonal antibody (Transduction Laboratories) 1:1000 in blocking solution for 60 min at RT. The membranes were then washed five times with PBS containing 0.1% Tween-20 before incubation with secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology Inc.) 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 1:5000 dilution of a mouse monoclonal antibody to β-actin (Sigma-Aldrich).

The relative signal intensity of CRADD (CASP2 and RIPK1 domain containing adaptor with death domain) and APAF-1 (apoptotic protease activating factor-1) protein detected in blots of tissue samples was quantified using Quantity One 1-D Analysis software (Bio-Rad Laboratories).

Immunohistochemistry

Four micrometer serial sections from routinely formalin fixed paraffin embedded blocks were cut for each case and mounted on poly-L-lysine coated glass slides. Standard deparaffinization and rehydratation of slides were performed. One section stained with H&E was used to confirm the histopatological diagnosis. Only sections showing sufficient epithelium to assess 1000 cells were considered for this study. Immunohistochemistry was then performed on the sections mounted on poly-L-lysine coated glass slides using labeled Streptavidin-biotin-alkaline phosphatase technique (LSAB-AP). To improve the staining pattern, the sections were boiled 3 times for 3 min in 10 mM citrate buffer as antigen retrieval method. In order to prevent non-specific binding sections were then preincubated with non-immune bovine serum (diluted 1:100 in PBS) for 25 min at RT. After 3 washes in Tris−HCl buffer, the slides were incubated overnight with the primary mouse monoclonal antibody against p120ctn (Transduction Laboratories) diluted 1:300 using 0.05 M Tris−HCl buffer pH 7.2-7.6, containing 1% bovine serum albumin. A negative control was performed in each run by substituting primary antibodies with non-immune reagent (Dako Antibody Diluent). Positive controls were performed in each run, using sections of normal skin/oral mucosa biopsies. Sequential 20-min incubations with biotinylated anti-mouse immunoglobulins and streptavidin conjugated to alkaline phosphatase were performed. Finally, fuchsine substrate system (K6998, Dako) was applied as a chromogenic solution. The slides were then counterstained with hematoxylin.

Statistical analysis

Data were compiled and analyzed in both a quantitative and semi-quantitative manner. The number of p120ctn expressing tumor cells was estimated as a percentage of the final number of 500 neoplastic cells of each case.

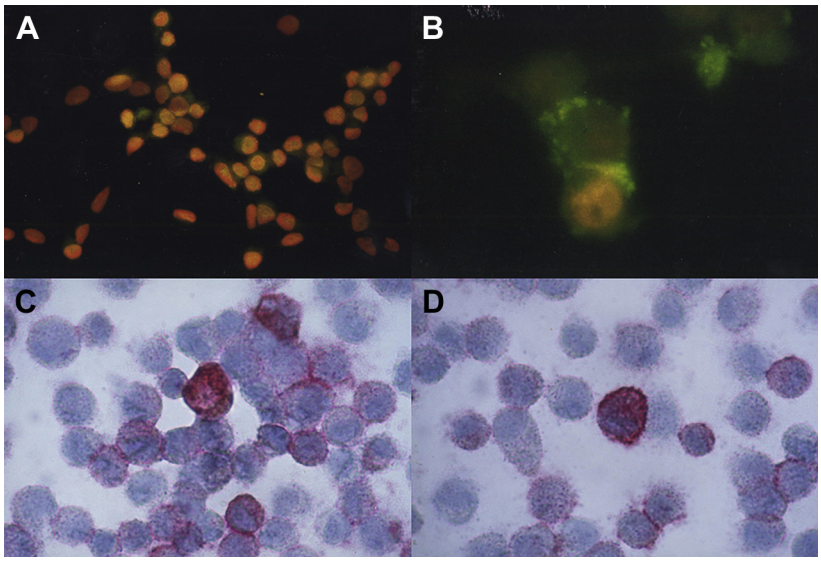

Fig. 1. (**A**) p120ctn localization in NCTC cell line by immunofluorescence: diffuse cytoplasmic staining and small well defined nuclear areas (×400); (**B**) Intracellular distribution of p120ctn in KM5 cell line: the staining was mostly diffuse, but was also associated with small punctate membranous structures at cell−cell contact and discrete dots in the nuclear region. (×1000, Nuclei were counterstained with Propidium Iodide); (**C**) Expression of p120ctn in OSC20 cell line (LSAB-AP, ×400); (**D**) Nuclear expression of p120ctn in KM5 cell line (LSAB-AP, ×400).

For the semi-quantitative analysis, four categories were identified including percentage and cellular localization of p120ctn: 0 (less than 10% of positive cells); 1 (>90% of normal membranous staining); 2 (>50% of cells with delocalized cytoplasmic staining or >3% nuclear staining). For the quantitative analysis, the percentage of p120ctn expression was evaluated in continuous scale of values ranging from 0 to 100%. Differences between groups were analyzed with the Student−Newman−Keuls test. Correlation by Pearson's method was also calculated to establish connection and to quantify the strength of the association between variables. Survival curves were analyzed according to the method of Kaplan−Meier and for differences between curves, the *P* value was calculated by the log-rank test. A *P* value of less than .05 was accepted as statistically significant.

RESULTS

p120ctn expression in oral cell lines

NCTC2544 showed a prevalent membranous positivity, even if polarized to cell−cell adhesion sites (Figure 1*A*). All neoplastic cell lines showed a prevalent cytoplasmic localization or no staining. CAL33 cells showed a membranous positivity in only 30% of cells, cytoplasmic positivity in 35% or no positivity in 35%. CAL27 showed a membranous positivity in only 20% of cells, cytoplasmic positivity in 40% or no positivity in 40%. OSC20 cells showed a membranous positivity in 40% of cells, cytoplasmic positivity in 20% or no positivity in 40%. KM5 cells showed a membranous positivity in only 10% of cells, cytoplasmic positivity in 40% or no positivity in 40%; in 10% of the cells there was a nuclear localization (Figure 1*B*, *C*, and *D*). Descriptive statistics for percentage of p120ctn expression in OSCC cell lines demonstrated a level of positivity ranging from 60% (CAL27, OSC20, KM5) to 65% (CAL33), showing no statistically significant difference between cytological degree of differentiation when compared with one-way analysis of variance (ANOVA) and Student−Newman−Keuls tests. However, in cancer cells, p120ctn showed an inverse relationship with the degree of differentiation for a progressive displacement of the signal toward the cytoplasm or nucleus in dedifferentiated cells, even though it was not statistically significant.

To confirm and better evaluate intracellular distribution of p120ctn, we probed the above cell lines by immunofluorescence. All cell lines showed diffuse staining for p120ctn. The protein localized both to cell−cell contacts, especially in NCTC2544 and OSC20, and in the cytoplasm. Furthermore, in all cell lines, several cells showed a nuclear localization, which was confirmed by DNA counterstaining with propidium iodide. In the nuclear region, p120ctn-related green fluorescence was appreciable as small, well-defined areas (Figure 1*A* and *B*).

Expression levels of p120ctn in oral cell lines were assayed by WB (Figure 2*A*). Anti-p120 Mab recognized bands ranging from 90 to 115 kDa and the protein was detectable in all cell lines. A reduced expression

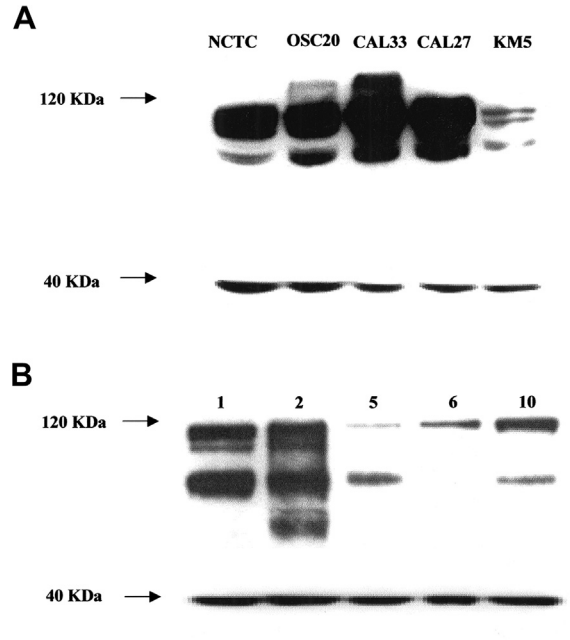

Fig. 2. WB showing expression levels of p120ctn (~120 KDa) and β-actin (40 KDa) in protein extracts from cell lines (**A**) and tissue samples (**B**). p120ctn was well expressed by all cell lines with the exception of KM5. There was a poor correlation between differentiation degree and expression pattern (**A**). Oral cancer expressed different levels of p120ctn with respect to normal mucosa. Poorly differentiated carcinomas (5, 6, 10) corresponded to a reduced expression of p120ctn. Well-differentiated carcinoma (2) showed no reduction in expression of protein compared to normal mucosa (1). Expression of β-actin was consistent among all samples (**B**).

of p120 was observed in KM5, while OSC20, CAL33, and CAL27, showed overexpression of the protein with respect to NCTC2544, a well-differentiated immortalized keratinocyte cell line. The intensity of β-actin protein was almost equal in all cell samples. These results suggested a poor correlation between p120ctn expression pattern and the degree of cellular differentiation, but examined expression levels only, without any distinction as to intracellular localization of the protein.

p120ctn expression in normal mucosa and OSCC

All 12 normal oral specimens showed homogenous membranous p120ctn localization (Figure 3*A* and *B*). Membranous staining was observed at the intercellular borders of histologically normal oral epithelium present in the OSCC specimens, although no staining was seen in the most superficial cells.

Abnormal staining patterns (negative, cytoplasmic, or nuclear) of p120ctn were seen in 96% (109/113) of cases. Complete loss of expression was found in 15% (21/113) of OSCC.

Well-differentiated OSCC (G1) showed a distribution pattern for p120 quite similar to that observed in normal epithelium. In fact, a high percentage of tumor cells showed a membranous staining pattern (Figure 3*C*). However, in several cases, predominantly G2, a lack of membrane localization for p120ctn was observed, since the signal was cytosolic (Figure 3*D* and *E*). Absence of membrane localization and cytosolic or nuclear staining for p120ctn were detected in poorly differentiated OSCC (G3) (Figure 3*F*).

Expression of p120ctn in tissue extracts of normal mucosa and OSCC was also demonstrated by WB (Figure 2*B*). p120ctn was detectable in all normal mucosa and OSCC but at different levels. Well-differentiated carcinomas showed similarly increased protein expression compared with normal mucosa, while the reduced expression was notable, as was the occasional lack of immunoreactive bands of p120ctn in poorly differentiated carcinomas.

Statistical analysis

There was a statistically significant difference ($P < .05$) in delocalization of p120ctn in OSCC's when compared with the control group. However p120ctn expression did not show statistically significant differences when compared with clinicopathological indices (Table I). Survival curves, comparing no expression of p120ctn (score 0) and normally membranous expression (score 1) and delocalized expression (score 2) by Kaplan−Meier method, showed statistically significant differences ($P < .05$) (Figure 4).

DISCUSSION

The p120ctn protein is a member of the catenin family.[7,29,30] It has been demonstrated that p120ctn binds to the same region of E-cadherin as the rest of the catenins, acting as a regulatory protein of the adhesive function of E-cadherin.[7,30] The interaction with juxtamembrane domain of cadherins allows p120ctn to affect cell adhesion, and has identified additional roles in the nucleus.[31] p120ctn goes into the nucleus,[32] but unlike β- and γ-catenin, it interacts with the transcription factor Kaiso, considered a possible transcription factor for p120ctn,[33] and a candidate downstream effector of cadherin and/or p120ctn signaling.[34]

Several studies have demonstrated that loss or reduction of p120ctn expression is frequently associated with high grade and advanced stage in a variety of malignancies,[20,35-37] including squamous cell carcinoma of the skin[19] and recently also OSCC.[22]

To best evaluate the correlation between p120ctn expression levels and cellular differentiation, cell lines were chosen taking the differentiation degree into account. All lines demonstrated immunoreactivity for

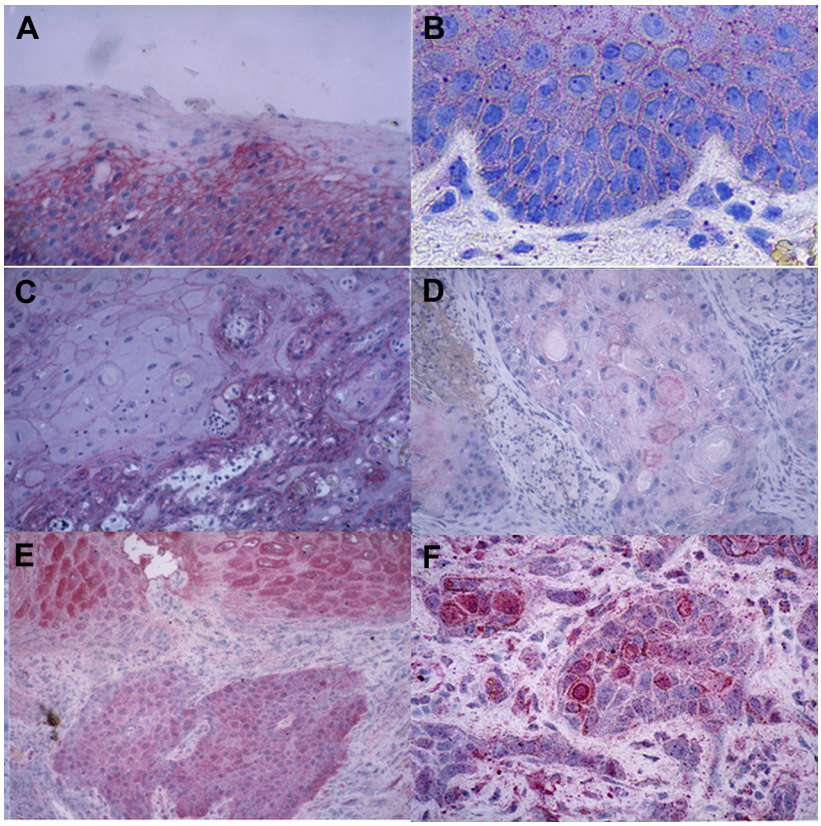

Fig. 3. (**A**) Normal pattern of staining for p120ctn in oral epithelium, with a definite cytoplasmic membrane localization (LSAB-AP, ×150); (**B**) Normal pattern of staining for p120 in oral epithelium; higher magnification (LSAB-AP, ×400); (**C**) Membranous staining for p120ctn in a case of G1/G2 OSCC, predominantly at the invasive front of the tumor (LSAB-AP, ×150); (**D**) Loss of the membrane staining for p120ctn in a case of G2 OSCC with a poor prognosis (LSAB-AP, ×250); (**E**) Cytoplasmic delocalization of immunopositivity for p120ctn in a case of G2 OSCC (LSAB-AP, ×106); (**F**) A case of G2/G3 OSCC: some cells showed a loss of the membrane staining for p120ctn with nuclear delocalization of the signal, while other cells showed a ring of cytoplasmic membrane and nuclear membrane positivity (LSAB-AP, ×400).

p120ctn by WB, but expression levels were not in agreement with the differentiation degree. In addition to total protein amount, it is likely that catenin localization must also be considered. NCTC2544 showed a membranous expression of the protein, especially at the site of cell−cell adhesion. Immunofluorescence confirmed a preferential localization to cell−cell contact and showed slight cytoplasmic staining. Abnormal staining patterns (either negative, heterogenous or cytoplasmic) of p120ctn were seen in OSCC cell lines (CAL27, CAL33, OSC20). High cytoplasmic expression of p120ctn is probably due to an increase of the free p120ctn fraction in tumor cells, similar to that of β-catenin.[38] A small number of neoplastic cells (CAL27, CAL33, OSC20, KM5) showed no positivity, while 30%-35% showed a reduction of expression. Absent or reduced catenin immunostaining in poorly differentiated tumor cells can be explained by a loss of their functions and structures. In fact the maintenance of tissue architecture depends on the function of cadherins,[39] which relies on interactions with catenins.

A total of 10% of KM5 cells, characterized by poorly differentiated keratinocytes, showed a nuclear positivity by immunocytochemistry. Immunofluorescence revealed a small fraction of cells showing a nuclear signal in all cell lines. Thus, p120ctn nuclear localization could suggest a potential involvement of this protein in signaling and eventually in cancer transformation, even if its role is still unknown.

These data were confirmed by the immunohistochemical evaluation. Abnormal staining patterns (negative, heterogenous, cytoplasmic, or nuclear) of p120ctn were seen in 96% (109/113) of cases. Complete loss of expression was found in 15% (21/113) of OSCC while normal oral epithelium showed membranous expression of p120ctn at the cell−cell borders, reflecting the normal localization of intercellular adhesion molecules, similar to that of β- and γ-catenins.[14,15,40]

All the cases in this study showed strong reactivity with p120ctn in areas of normal epithelium (Figure 3*A* and *B*). A similar pattern of staining was observed in all

Table I. Statistical analysis of p120ctn score expression and associated clinicopathological findings in oral SCCs

Variables	*No.*	*Score 0 n.*	*Score 1 n.*	*Score 2 n.*	*Mean*	*Standard deviation*	*Standard error*	P *< .05*	*Statistical data*			
Cases	113	21	4	88								
Age												
≤65 years	59	10	3	46	1.610	0.7660	0.09973	No*		$P = .9339$		
>65 years	54	11	1	42	1.574	0.8150	0.1109					
Sex												
Male	77	12	4	61	1.500	0.8783	0.1464	No*		$P = .3747$		
Female	36	9	0	27	1.636	0.7418	0.08453					
Grading										Mean difference	*q*	*P* value
G1	39	8	0	31	1.590	0.8181	0.1310	No	G2 vs. G3	−0.03414	0.2360	>.05
G2	53	9	4	40	1.585	0.7705	0.1058		G2 vs. G1	−0.00483	–	>.05
G3	21	4	0	17	1.619	0.8047	0.1756		G1 vs. G3	−0.02930	–	>.05
Size												
<1.5 cm	50	9	1	40	1.620	0.7796	0.1103	No*		$P = .7460$		
>1.5 cm	63	12	3	48	1.571	0.7975	0.1005					
Lymph node metastasis												
Negative	87	15	2	70	1.632	0.7642	0.08194	No*		$P = .3339$		
Positive	26	6	2	18	1.462	0.8593	0.1685					
Staging										Mean difference	*P* value	
I	53	9	1	43	1.642	0.7619	0.1047		Stage III vs. stage IV	−0.2724	>.05	
II	24	5	1	18	1.542	0.8330	0.1700	No†	Stage III vs. stage I	−0.2297	>.05	
III	17	4	2	11	1.412	0.8703	0.2111		Stage III vs. stage II	−0.1299	>.05	
IV	19	3	0	16	1.684	0.7493	0.1719		Stage II vs. stage IV	−0.1425	>.05	
									Stage II vs. stage I	−0.0998	>.05	
									Stage I vs. stage IV	−0.0427	>.05	
Recidive												
Yes	40	7	1	32	1.525	0.8469	0.1339	No*		$P = .4992$		
No	73	14	3	56	1.630	0.7547	0.08834					

*Student−Newman−Keuls' test.
†One-way analysis of variance (ANOVA) and Student−Newman−Keuls multiple comparisons test.

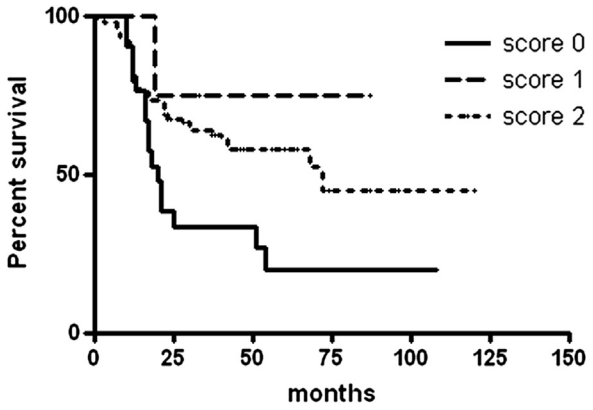

Fig. 4. Survival curves, comparing low/no expression of p120ctn (score 0), normal membranous expression (score 1) and delocalized expression (score 2) by Kaplan−Meier method, show statistically significant differences ($P < .05$).

well-differentiated OSCC cases in our series. In contrast, the infiltrating areas of moderately differentiated tumors showed a lower or absent signal, with a general reduction of staining intensity at the peripheral border of cancer nests. Poorly differentiated OSCC showed a homogenous decrease and/or a total lack of immunostaining, even if some cases showed nuclear positivity (Figure 3*C-F*). The reduction of expression and the altered localization of p120ctn are very common events during the development and/or progression of SCCs.[19]

The similarity in staining pattern between normal squamous epithelium and well-differentiated OSCC of our series supports the existence of a possible relationship between p120ctn expression and the degree of cellular differentiation. This is further supported by the finding of low or absent membranous immunopositivity both in poorly differentiated OSCC and at the invasive front of moderately OSCC. This data was confirmed by WB which evidenced a reduction of p120ctn expression in poorly differentiated OSCC with loss of immunoreactive bands in some samples, while well differentiated OSCC showed the same pattern, or in some instances, a overexpression of the protein with respect to normal mucosa.

Analysis of p120ctn expression in OSCC might be useful in identifying tumor areas with more aggressive potential in well and moderately differentiated tumors, in addition to the study of E-cadherin expression and other conventional markers such as those related to

cellular proliferation. Other parameters were less reliable, such as the pattern of invasion, which did not show any correlation with the degree or with the topography of the immunopositivity.

Up-regulation of p120ctn cytoplasmic staining, already described for other tumors,[41] was also seen in OSCC. High cytoplasmic expression of p120ctn is probably due to an increase of the free p120ctn fraction in tumor cells, and could suppose a potential involvement in cancer transformation.[42] Furthermore, altered localization of this protein is prognostic for aggressive disease in some tumors, like breast or colon carcinoma.[17,20] The exact role of p120ctn is not yet completely clear, but this molecule seems to have an unexpected dual function, promoting tumor suppression or inducing neoplastic transformation, depending on whether E-cadherin is present or not.[43]

The E-cadherin-catenin complex is the mediator of intercellular cohesion and epithelial tissue integrity. Epithelial-mesenchymal transition (EMT) is the process by which epithelial cells adopt a mesenchymal phenotype or fibroblast-like properties, reorganizing their cytoskeleton and breaking connections with their neighbors, and is suggested to play an important role during cancer invasion and metastasis. The best-studied process of EMT is "cadherin switching," involving the inhibition of E-cadherin expression, and an aberrantly high expression of N-cadherin.[44] The disturbance in E-cadherin-p120ctn leads to accumulation in the cytoplasm of this protein, confirmed by the abnormal staining pattern. It has been demonstrate that p120ctn can down-regulate the enzymatic activity of GTPase enzymes that regulates actin assembly, causing instability of intercellular adhesion and inducing cell migration through the formation of lamellipodia and filopodia.[45] These findings lead to candidate p120ctn as another protein for the control of the metastatic potential of tumors, as down-regulation of adhesion is a primary event in metastasis.[46,47] However, p120ctn shows heterogenous expression pattern, with isoform variability, but no evidence of complete loss of expression in tumor cell lines, such as other adhesion proteins.[48]

Abnormal staining pattern of p120ctn can reflect reduction or loss of adhesion, as in other human malignancies. The strong correlation seen in OSCC between loss of membranous immunoreactivity and grade suggests that alterations in E-cadherin-p120ctn complex expression and function play an important role in OSCC tumor progression. Several reports have shown that loss of membranous E-cadherin expression in OSCC is associated with advanced stage and progression.

To date, no studies have found a correlation between p120ctn expression in OSCC and patient survival. In this study. Data showed an inverse association between the percentage of stained cells and the survival rate. Indeed, the results indicated that patients with loss of p120ctn expression had poorer survival rates than the group with normal p120ctn expression ($P < .05$). Moreover, cases presenting p120ctn cellular delocalization showed a worse survival rates than the group with normal p120ctn expression. When p120ctn and others clinicopathological features, such as grading and staging, were analyzed for prognostic significance, no statistically significant correlations were detected. Thus, the level of p120ctn expression could be considered as an independent prognostic factor. This finding was confirmed by a very recent work that displayed how in potentially malignant oral lesions the mislocalisation of expression of p120ctn from the cell membrane to the cytoplasm is associated with oral cancer progression.[22] Stairs et al. reported that in a conditional knockout model of p120ctn in mice, the tumor-derived cells secrete granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), monocyte chemotactic protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α). These cytokines create a favorable tumor microenvironment with inflammatory and tumorigenic features, and with a significant percentage of immature myeloid cells, that have the ability to suppress antigen-specific T cell activation. These results suggest that p120ctn can be considered "de facto" a tumor suppressor gene.[49]

In conclusion, we have shown that loss of physiological membranous p120ctn immunoreactivity occurs in 96% of OSCCs, and, as already reported for E-cadherin, β- and γ-catenin, the reduction or the absence of p120ctn expression was correlated to poor prognosis.

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