

E-cadherin, β -catenin, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin expression in primary oral squamous cell carcinoma and its regional metastasis

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Summary. To investigate E-cadherin, β -catenin, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in 40 samples of non-metastatic and metastatic oral squamous cell carcinoma (OSCC) with positive cervical lymph nodes (LN). Immunohistochemistry was used to evaluate expression in the lesion center (LC) and invasive tumor front (ITF) of non-metastatic (n=18) and metastatic (n=22) OSCC and in the LN on the metastatic neoplastic cells (MNC; n=22). In metastatic OSCC, E-cadherin and β -catenin presented significantly lower cytoplasmic membrane expression in the ITF and MNC when compared to the LC and lower cytoplasmic expression in MNC when compared to the LC and ITF ($p < 0.05$). Integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ showed high cytoplasmic expression in the LC, ITF and MNC ($p > 0.05$). A positive correlation was observed between E-cadherin cytoplasmic expression and $\alpha 2\beta 1$ ($\rho = 0.860$) and $\alpha 3\beta 1$ ($\rho = 0.975$) expression. When comparing the primary sites of metastatic and non-metastatic disease, β -catenin presented lower cytoplasmic membrane ($p = 0.013$) expression in metastatic OSCC. E-cadherin presented low expression and the integrins high expression in both groups. Abnormal expression of β -catenin and E-cadherin associated with high expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins contribute to LN metastasis in OSCC.

Key words: Oral cancer, Squamous cell carcinoma, Cell adhesion, Lymphatic metastasis

Introduction

E-cadherin (E-cad), a cell adhesion molecule expressed in epithelial tissues, is a transmembrane glycoprotein that promotes cell adhesion through calcium-dependent binding to the extracellular domain of E-cad molecules in adjacent cells (Larue et al., 1996). This function depends on association with β -catenin (β -cat), a multifunctional cytoplasmic protein, which, through its association with α -catenin, creates a link between E-cad and the actin cytoskeleton (Xu and Kimelman, 2007).

Previous investigations have shown that the loss of E-cad cytoplasmic membrane expression is associated with lymph node metastasis, high invasiveness, recurrence and poor prognosis (Kudo et al., 2004; Gould Rothberg and Bracken, 2006; Hong et al., 2011; Carneiro et al., 2012) while cytoplasmic and nuclear expression have also been reported in several malignancies (Audard et al., 2008; Chetty et al., 2008). The reasons why E-cad presents cytoplasmic and nuclear expression in some neoplastic cells are not clear, nor its significance in these locations. However it is believed that it contributes to tumor progression (Ferber et al., 2008; Kaur et al., 2009; Vered et al., 2012).

When E-cad is linked in normal keratinocytes, the β -cat is expressed in cell membrane and its cytoplasmic expression is strongly suppressed by degradation of the

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complex formed by Axin protein that binds the adenomatous polyposis coli (APC) and glycogen synthase kinase 3 β - (GSK3- β) (Aberle et al., 1997). The loss of membranous expression and cytoplasmic and nuclear accumulation of β -cat have been reported in different malignant tumors (Chesire et al., 2000; Gao et al., 2005; Cai et al., 2008; Freitas Rde et al., 2010; Xu et al., 2012; Han et al., 2013). Furthermore, β -cat participates in two crucial pathways involved in the progression of neoplasms: the Wntless-wnt signaling pathway (Wnt), a powerful regulator of cell proliferation and differentiation (Nelson and Nusse, 2004; Pannone et al., 2010), and the epithelial-mesenchyme transition (EMT) pathway, which induces a mesenchymal phenotype in cells of epithelial origin, thereby inducing a loss of E-cad expression and contributing to invasion and metastasis (Brabletz et al., 2005; Guarino, 2007; Nakamura and Tokura, 2011). However, OSCC investigations concerning the relationship between the immunoexpression of E-cad, β -cat and the occurrence of metastasis have shown conflicting results (Cai et al., 2008; Freitas Rde et al., 2010; Vered et al., 2012; Zhao et al., 2012).

Integrins are the most predominant and well characterized transmembrane receptors in several extracellular matrix proteins, including fibronectin, laminin, vitronectin and collagen-IV (Weber et al., 2011; Ganguly et al., 2013). Integrins play key roles in many biological processes including actin cytoskeleton organization and transduction of intracellular signals regulating cellular functions such as cell growth, apoptosis, cell division, and leukocyte migration in inflammation (Cabrijan and Lipozencic, 2011). Among the abundant constitutive integrins in epidermis are $\alpha 2\beta 1$ and $\alpha 3\beta 1$, which are respectively a collagen and laminin receptor and a laminin-5 receptor (Watt, 2002). The participation of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in carcinomas progression is well documented, furthermore alterations in immunoexpressions of these integrins (α and β subunits) have been associated to invasion and metastasis (Hashida et al., 2002; Shield et al., 2007; Ohara et al., 2009; Mitchell et al., 2010; Ramirez et al., 2011; Haidari et al., 2012).

Interactions between E-cad, β -cat and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins has already been reported in the literature (Whittard et al., 2002; Chattopadhyay et al., 2003; Kim et al., 2009). In squamous cell carcinoma, the loss of intercellular adhesion mediated by E-cad was previously linked to the elevated cell surface expression of $\alpha 2$, $\alpha 3$ and $\beta 1$ integrins, associated with the conversion of premalignancy to cancer in keratinocyte cell lines (Zhang et al., 2006). Nevertheless, despite evidence implicating $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in malignancy progression, little is known about their roles in tumorigenesis or how they regulate malignant cell behavior. In addition, few studies have investigated these integrins in OSCC (Ohara et al., 2009; Amaral Pereira et al., 2013) and there are no data in recent literature concerning their expression in metastatic neoplastic cells (MNC) in

cervical lymph nodes. Furthermore, there are no studies investigating the expression of E-cad, β -cat, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in OSCC in the same sample in different tumor microenvironments.

The invasive tumor front (ITF) is the tumor-host interface, where several molecular events of importance to tumor spread occurs. Substantial data strongly suggest that neoplastic cells in the ITF differ considerably from neoplastic cells in the lesion center (LC), which corresponds to the most superficial area of the tumor (Bankfalvi and Piffko, 2000; Mahomed et al., 2007; Wang et al., 2009) (Fig. 1). The ITF might consist of more aggressive cells and metastatic clones probably reside in this area (Bryne, 1998; Bankfalvi and Piffko, 2000; Mahomed et al., 2007). Hence, the aim of this study was to investigate the immunoexpression of E-cad, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in OSCC with and without lymph node metastasis in LC, ITF and in the respective metastatic cervical lymph nodes.

Materials and methods

Samples

This study was approved by the Federal University of Goiás Research Ethics Committee for human subjects, and registered as number 339/11. The samples consisted of surgically excised specimens from 40 patients with primary OSCC obtained from the Anatomopathology Division of the Araújo Jorge Hospital, Goiás Combat Cancer Association, Brazil. This study used paraffin blocks in good condition with sufficient tissue for analysis taken from 40 OSCC patients subjected to surgery from 2001 to 2004. Patients with squamous cell carcinoma in other sites, those with no clinical follow-up, and those who had received radiotherapy, chemotherapy, or any other treatment before surgery were excluded from the study. The clinical data and follow-up information (recurrence, survival time and death) were obtained from medical records. Three groups were formed from the samples selected: 1 - primary OSCC without metastatic cervical lymph nodes (n=18); 2 - primary OSCC with cervical lymph node metastasis (n=22); and 3 - positive cervical lymph node samples (n=22) from group 2.

Light microscopy

Specimens were fixed in 10% buffered formalin (pH 7.4) and paraffin embedded. Microscopic features were evaluated based on one 5- μ m section of each sample, stained with hematoxylin and eosin. Two pathologists (A.C.B. and R.C.G.A.) evaluated all the serial sections of the lymph nodes simultaneously using light microscopy to confirm the presence or absence of lymph node metastasis and to characterize the OSCC. All OSCC sections were graded according to the World Health Organization (WHO) classification of tumors (Pindborg et al., 1997), and the presence of

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lymphovascular permeation and perineural invasion was evaluated.

Immunohistochemistry technique

Samples investigated in this study were tested for mouse monoclonal E-cadherin (clone SPM471, epitope Aa 600-707, Spring Bioscience, Pleasanton, CA, USA; dilution 1:200); mouse monoclonal β -catenin (clone E-5, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100); mouse monoclonal Integrin $\alpha 2\beta 1$ (clone BHA2.1 Chemicon International, TemelUCA, CA, USA; dilution 1:50) and mouse monoclonal Integrin $\alpha 3\beta 1$ (clone M-KID2 Chemicon International, TemelUCA, CA, USA; dilution 1:50). Paraffin-embedded tissues were sectioned (3 μ m) and collected in series on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma Aldrich, St. Louis, MO, USA). The sections were deparaffinized in xylene, rehydrated in decreasing concentrations of alcohol and washed in a buffer saline solution (PBS) (pH 7.2). Then the sections were immersed in warm water and immediately incubated in citrate buffer (pH 6.0) (for anti-E-cad and anti-Integrin $\alpha 3\beta 1$), or in Tris- EDTA buffer (pH 9.0) (for anti- β -catenin) at 95°C in a digital water bath for 20 minutes; or with pepsin for 30 minutes in a 37°C oven (for anti-Integrin $\alpha 2\beta 1$) for antigen retrieval. The sections remained immersed in the solution for progressive cooling for 10 min and were then washed in PBS. Afterwards, they were immersed in 3% hydrogen peroxide diluted in PBS for 30 min, washed and incubated in a blocking reagent. They were subsequently subjected to the primary antibody reaction in a moist chamber overnight at 4°C. After washing in PBS, the sections were treated with MACH4- Universal HRP-Polymer kit (BioCare, Concord, CA, USA) for anti-E-cadherin; a streptavidin-biotin immunoperoxidase method (LSAB kit, Dako, Carpinteria, CA, USA) for anti- β -catenin; and the Catalyzed Signal Amplification System (Dako Carpinteria, CA, USA) for anti- $\alpha 2\beta 1$ integrin and anti- $\alpha 3\beta 1$ integrin in accordance with the supplier's protocol. The slides were then incubated in 3,3'-Diaminobenzidine in a chromogen solution (Dako) for 2 to 5 min at room temperature. Finally, the sections were stained with Mayer's hematoxylin for 20 seconds, washed in running water for 5 min, dehydrated in increasing concentrations of alcohol, incubated in xylene twice and then covered. The positive control was fibroepithelial hyperplasia from oral mucosa and the negative control was obtained by omitting the primary antibody, which was substituted by 1% PBS-BSA solution.

Evaluation of immunoreactivity

Staining was evaluated at the primary site in the LC and ITF and in positive lymph nodes in MNC using a x400 magnification lens in a multi-view microscope. The evaluation was performed simultaneously by two

examiners who were unaware of the clinical and morphological data. Occasional disagreements were discussed until consensus was reached.

For the qualitative evaluation, the pattern of E-cad and β -cat expression was considered positive when membranous (cytoplasmic membrane), cytoplasm and/or nucleus staining was observed. The membranous immunostaining for E-cad and β -cat was considered positive only when continuous membranous staining was observed, regardless of the intensity of staining. Integrin expression was considered positive when present in the neoplastic cell, regardless of cell region or staining intensity.

A semi-quantitative analysis was performed, five alternate fields were evaluated and the samples were classified into two categories: low expression (0% to 50%) and high expression (51% to 100%) of cells showing positive staining (Kurtz et al., 2006; Foschini et al., 2008; Ohara et al., 2009).

Statistical analysis

The statistical analysis was performed using SPSS 17.0 software. Firstly, a descriptive analysis of clinicopathological features was performed. Chi-square and Fisher's Exact tests were used for between-groups

Table 1. Clinical and pathological data on the patients.

Clinical and microscopic features		Patients (%)
Age	<50	37.5
	\geq 50	62.5
Gender	Male	62.5
	Female	37.5
Tobacco	Yes	82.5
	No	17.5
Alcohol consumption	Yes	70
	No	30
Location	Oral tongue	52.5
	Floor of the mouth	32.5
	Others	15
T stage*	T1-T2	28.9
	T3-T4	71.1
Clinical outcome	Dead	60
	Alive (overall survival)	40
Lymph node metastasis	Yes	22
	No	18
Recurrence	Yes	17
	No	23
Histological grading (WHO grade)	I-II	82.5
	III	17.5
lymphovascular permeation	Yes	40
	No	60
Perineural invasion	Yes	25
	No	75

* 2 missing cases.

comparison regarding protein expression and histological features. The correlation in the expression patterns between E-cad, β -cat, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ was assessed using Spearman's test. Kaplan-Meier analysis and Cox regression were used to investigate factors affecting patient survival. A level of significance of 0.05 ($p < 0.05$) was adopted for all tests.

Results

Clinical findings

An assessment of 40 patients with OSCC (22 with cervical lymph node metastasis and 18 without) revealed an age range varying from 24 to 90 (mean=57) and a predominance of males (62.5%). Details concerning description of clinical and microscopic findings are summarized in Table 1.

Kaplan-Meier analysis showed that overall patient survival proportion was 40% (mean survival=57.8 months; 95%CI=37.9-77.6). Significant difference (log-rank test - $p=0.023$) was found between survival in non-metastatic (mean=80.3; 95%CI=51.8-108.7) and metastatic OSCC patients (mean=38.3; 95%CI=15.4-61.2). Cox regression showed that from all clinicopathological variables, only lymph node metastasis was associated with survival (HR=2.67; 95%CI=1.11-6.43; $p=0.028$).

Association of clinicopathological findings with the expression of E-cad, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$

Only tumor recurrence was associated with high expression of $\alpha 3\beta 1$ in the ITF ($p=0.004$), since all patients who presented recurrence also presented high

expression of $\alpha 3\beta 1$ integrin. No significant association was observed between protein immunorexpression and tumor size, histological grading, perineural invasion, lymphovascular permeation or survival.

The E-cad, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ expression pattern in metastatic OSCC and in MNC

The immunohistochemical expression of the neoplastic cells from group 2 was compared with that of group 3 (MNC). The results are summarized in Table 2. There was a significant progressive loss of expression in the cytoplasmic membrane of E-cad and β -cat from the normal epithelium to LC and from LC to ITF and this low expression of E-cad and β -cat was also observed in the MNC. When the expression of these molecules in the cytoplasm was analyzed, high expression was seen in the primary tumoral parenchyma and lower expression in the MNC (Figs. 2, 3).

In the normal epithelium the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins showed low staining in cytoplasmic membrane and no staining was observed in cytoplasm, while predominantly high cytoplasmic immunostaining was observed in the LC, ITF and MNC (Figs. 4, 5).

The E-cad, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ expression patterns in non-metastatic and metastatic primary OSCC

In the LC the percentage of sections showing low expression of E-cad in the cytoplasmic membrane was higher for group 2 than for group 1, although there was no statistically significant difference between the groups, while in the ITF both groups showed low expression of E-cad. In groups 1 and 2, high cytoplasmic expression of E-cad was predominantly seen in the LC and ITF. Nuclear expression was predominantly low in the LC and ITF in both groups.

Table 2. Qui-square test for expression of proteins in metastatic OSCC and in MNC.

		LC (%)	ITF (%)	MNC (%)	p- value
E- cad (M)	Low	72.7	100	95.5	0.007*
	High	27.3	0	4.5	
E-cad (C)	Low	22.7	13.6	50	0.021*
	High	77.3	86.6	50	
E-cad (N)	Low	86.4	100	91	0.220
	High	13.6	0	9	
β -cat (M)	Low	86.4	100	100	0.043*
	High	13.6	0	0	
β -cat (C)	Low	4.5	31.8	54.5	0.002*
	High	95.5	68.2	45.5	
β -cat (N)	Low	100	100	100	-----
	High	0	0	0	
$\alpha 2\beta 1$	Low	0	10	20	0.329
	High	100	90	80	
$\alpha 3\beta 1$	Low	30	50	20	0.350
	High	70	50	80	

M, membranous; C, cytoplasmic; N, nuclear. * Significant p-value < 0.05 .

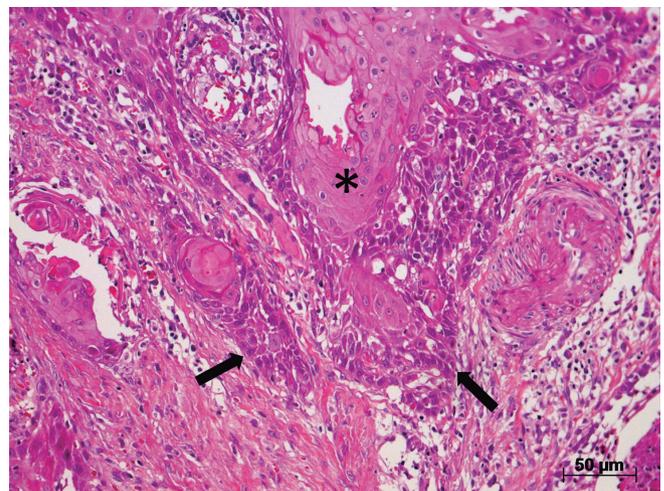


Fig. 1. Oral squamous cell carcinoma showing the lesion center (asterisk) and the invasive tumor front (arrows). Hematoxylin and eosin, x 200

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The immunoexpression of β -cat in the cell membrane was significantly lower in group 2 than in group 1 in the LC ($p=0.013$), while in the ITF low expression was observed in both groups 1 and 2. Cytoplasmic expression was predominantly high in groups 1 and 2 in the LC and ITF. Nuclear expression was low in both groups in both the LC and ITF.

Integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$ presented high cytoplasmic staining. There was no significant difference between the

integrin expression in groups 1 and 2 in the LC or in the ITF. These results are summarized in Table 3.

Correlation of expression patterns between E-cad, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins

A strong positive correlation was observed between E-cad expression in the cytoplasm and $\alpha 3\beta 1$ ($r=0.860$; $p=0.001$) and $\alpha 2\beta 1$ expression ($r=0.975$; $p<0.001$) in metastatic OSCC (group 2).

Discussion

The major determinant of prognosis in cases of OSCC is cervical metastasis (Noguti et al., 2012). Our data corroborate this, showing that the metastatic group presented a low survival rate. Metastasis is a complex process in which multiple interactions between tumor cells and host are required (Feller et al., 2012). In order to better understand the involvement of adhesion molecules in the metastatic process in OSCC, we investigated E-ca, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin expression in the LC, ITF and MNC in cervical lymph nodes.

Our findings demonstrate lower expression of E-cad and β -cat in the cytoplasmic membrane in the ITF region when compared to that of the LC. This is consistent with previous investigations (Bankfalvi et al., 2002; Mahomed et al., 2007; Wang et al., 2009) and demonstrates that the loss of expression of these molecules is more pronounced in areas of major tumor invasiveness. E-cad and β -cat loss was also observed in MNC and these findings corroborate the results reported by Kaur et al. (2009) and Cai et al. (2008) who reported negative expression of E-cad and β -cat in lymph nodes. Such observations represent further evidence of the contribution of the loss of expression of E-cad and β -cat in the cytoplasmic membrane to the migration and survival of tumor cells in lymph nodes.

E-cad and β -cat also presented high cytoplasmic expression at the center and invasive front of the tumor

Table 3. E-cad, β -cat, $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins expression pattern in non-metastatic and metastatic OSCC.

	Lesion Center (%)			Invasion Tumor Front (%)		
	Group 1	Group 2	p-value	Group 1	Group 2	p-value
E-cad(M)						
Low	61.1	72.7	0.435 ^a	100	100	----
High	38.9	27.3		0	0	
E-cad (C)						
Low	11.1	22.7	0.297 ^b	22.2	13.6	0.383 ^b
High	88.9	77.3		77.8	86.4	
E-cad (N)						
Low	88.9	86.3	0.598 ^b	100	100	----
High	11.1	13.7		0	0	
β -cat (M)						
Low	50	86.3	0.013 ^{a*}	100	100	----
High	50	13.7		0	0	
β -cat (C)						
Low	22.2	4.5	0.115 ^b	33.3	31.8	0.919 ^a
High	77.8	95.5		66.7	68.2	
β -cat (N)						
Low	83.3	100	0.083 ^b	100	100	----
High	16.7	0		0	0	
$\alpha 2\beta 1$						
Low	0	0	----	10	10	0.763 ^b
High	100	100		90	90	
$\alpha 3\beta 1$						
Low	40	30	0.500 ^b	30	50	0.325 ^b
High	60	70		70	50	

Group 1: non-metastatic OSCC; Group 2: metastatic OSCC; M, membranous; C, cytoplasmic; N, nuclear; a X2 Test; b Fisher's Exact test. * significant p-value <0.05.

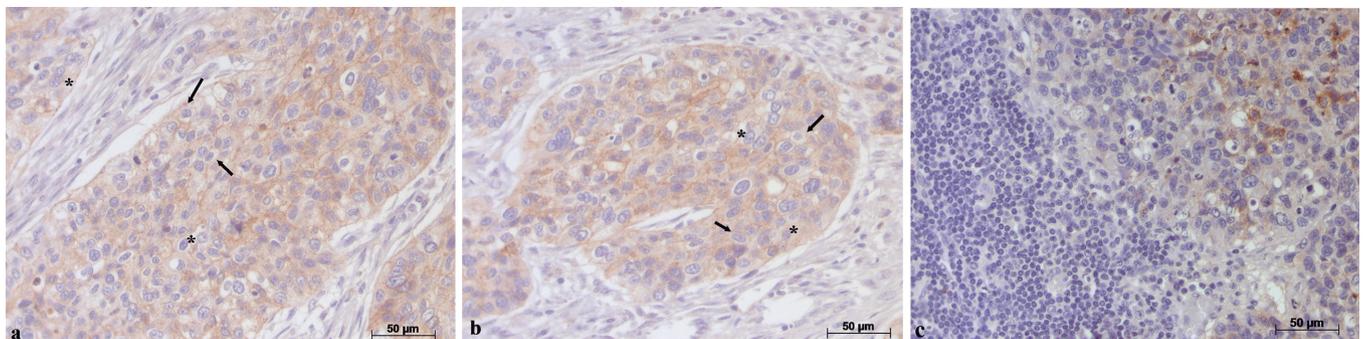


Fig. 2. E-cad immunoexpression in LC (a) and ITF (b) presented low cytoplasmic membrane expression (arrows), high cytoplasmic expression and low nuclear expression (asterisks). In MNC (c) E-cad showed Low expression in cytoplasmic membrane, cytoplasm and nucleus. x 400.

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and there was a loss of expression in MNC. Low expression of E-cad in the cytoplasmic membrane and high expression in the cytoplasm are indicative of the abnormal behavior of the neoplastic cell in OSCC (Kaur et al., 2009). Cytoplasmic expression of E-cad has also been associated with loss of cell differentiation in OSCC (Gao et al., 2005; Kaur et al., 2009). According to Kaur

et al. (2009), there are several ways of explaining the cytoplasmic immunoeexpression of E-cad. These include an increase in production, failure in translocation of this protein and anchorage flaws in the cell membrane (Kaur et al., 2009). The abnormal cytoplasmic expression of β -cat observed in our study is consistent with the findings of Cai et al. (2008). This kind of cytoplasmic expression

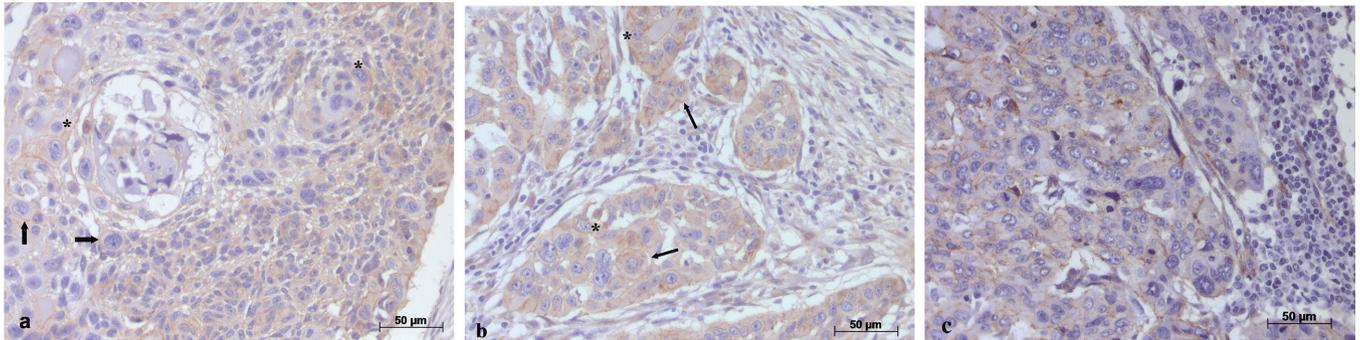


Fig. 3. β -cat immunoeexpression in LC (a) and ITF (b) presented low cytoplasmic membrane expression (arrows), high cytoplasmic expression and low nuclear expression (asterisks). In MNC (c) β -cat showed Low expression in cytoplasmic membrane, cytoplasm and nucleus. X 400.

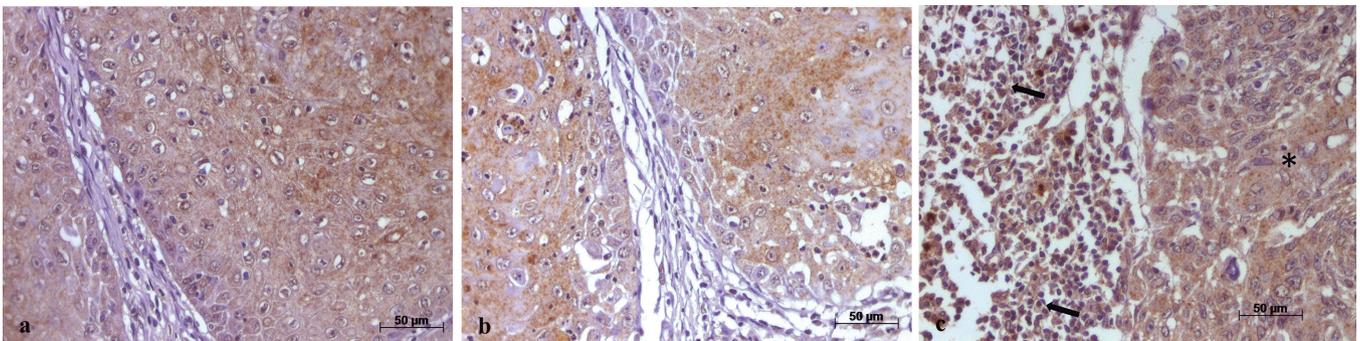


Fig. 4. The $\alpha 2\beta 1$ integrin presented high cytoplasmic immunoeexpression in LC (a), ITF (b) and MNC (c). In lymph nodes positive immunostaining was observed in neoplastic cells (asterisks) and in lymphocytes (arrows). x 400.

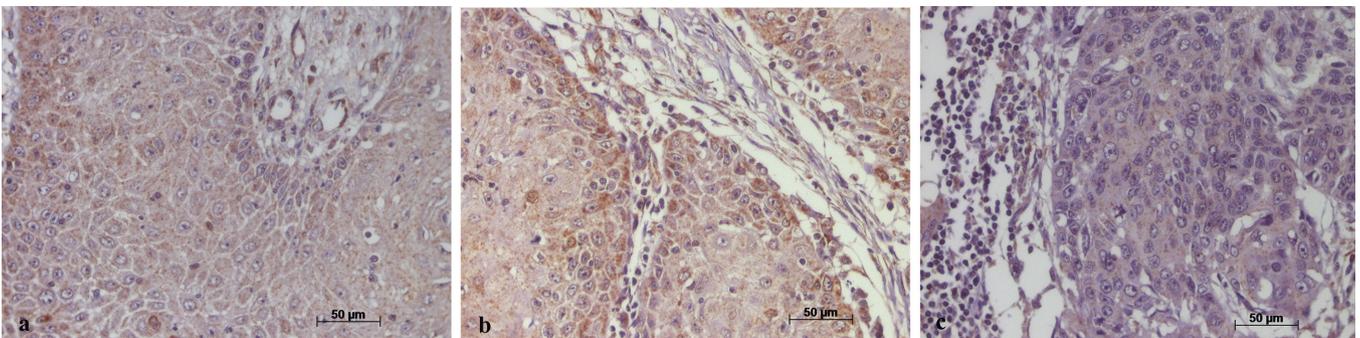


Fig. 5. The $\alpha 3\beta 1$ integrin presented high cytoplasmic immunoeexpression in LC (a), ITF (b) and MNC (c). x 400.

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has previously been linked to β -cat translocation to the nucleus, where it is known to contribute to tumor metastasis (Nelson and Nusse, 2004; Xu and Kimelman, 2007).

Although the nuclear expression of E-cad and β -cat was observed in our sample, it was predominantly low in the LC, ITF and MNC. Nuclear expression of these immunohistochemical markers is not observed in normal keratinocytes, but this expression has been reported in several carcinomas (Han et al., 2000; Serra et al., 2007; El-Bahrawy et al., 2008; Ronkainen et al., 2010; Zulehner et al., 2010; Le Bras et al., 2011; Ohishi et al., 2011). The nuclear expression of E-cad might contribute to neoplasm progression as an anti-apoptotic factor (Ferber et al., 2008), while β -cat participates in the transcription of Wnt pathway target genes and the EMT pathway (Le Bras et al., 2011; Swaminathan et al., 2012; Lu et al., 2013).

In terms of the integrins $\alpha 2\beta 1$ and $\alpha 3\beta 1$, high cytoplasmic expression was found in the LC and ITF. Amaral-Pereira et al. (2013) also observed high cytoplasmic expression and suggested that this abnormal expression is probably due to increased expression of these proteins when compared with normal keratinocytes, suggesting that not only is their adhesion function required by neoplastic cells, but also their activity related to cell proliferation and survival in ITF. Nevertheless, these authors just investigated the integrins expression in ITF without take into consideration the LC.

To our knowledge, this is the first study to investigate the expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ in OSCC in a primary site and in MNC. High levels of positive staining of $\alpha 2\beta 1$ were also seen in lymphocytes. These findings were expected since the expression of this integrin in lymphocytes, where it operates in inflammatory responses, has already been reported (McCall-Culbreath et al., 2008; Boisvert et al., 2010). High cytoplasmic expression of both integrins was observed in MNC. This could indicate that $\alpha 2\beta 1$ and $\alpha 3\beta 1$ actively contribute to cell migration in lymph node metastasis, as reported by Fennewald et al. (2012), who identified the $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins as specific receptors that mediate the interaction between tumor cells and laminin under conditions consistent with the lymphodynamic flow in head and neck squamous cell carcinoma. This suggests that these interactions could be critical for tumor cell growth and survival within the lymph node microenvironment (Fennewald et al., 2012).

We also explored the expression of the adhesion molecules in primary OSCC without metastatic cervical lymph nodes and primary OSCC with cervical lymph node metastasis. In LC metastatic carcinomas presented significantly lower expression of β -cat in the cytoplasmic membrane than non-metastatic carcinomas. An association between low membranous expression of β -cat and lymph node metastasis in OSCC has previously been reported (Bankfalvi et al., 2002; Tanaka et al., 2003; Cai et al., 2008). This association could be

attributed to the fact that the β -cat expressed in the cell membrane is linked to E-cad and is essential for its function and for cell adhesion. Hence, genetic mutations of β -cat, despite the normal expression of E-cad, contribute to the loss of cell adhesion, cell motility and migration (Oyama et al., 1994).

E-cad expression in the cytoplasmic membrane was lower in the metastatic than in the non-metastatic group, although the difference was not statistically significant. These findings have also been reported by Mahomed et al. (2007), but contrast with other studies in OSCC (Diniz-Freitas et al., 2006; Foschini et al., 2008). Additionally, we observed high E-cad cytoplasmic expression and low nuclear expression in OSCC in both groups with no statistical difference between the two. According to Vered et al. (2012), the controversy about E-cad immunoexpression and its potential as a prognostic marker is probably due to the diversity of methodologies used to assess its immunoexpression and due to the tumor dynamic whereby E-cad expression is not dictated solely by carcinomatous cells but is influenced by the tumor microenvironment.

No differences in $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrin staining were observed between non-metastatic and metastatic groups, although an association between these integrins and metastasis has been reported in OSCC and other carcinomas (Shield et al., 2007; Ohara et al., 2009; Ramirez et al., 2011). Ohara et al., reported that patients who presented metastasis also presented a significant decrease of $\alpha 3$ and $\beta 1$ integrin in ITF, however in this investigation the samples were from pre-treatment biopsy and the presence of metastasis were established based on clinical data, different from ours in which the samples were obtained from complete tumor resection and the metastasis diagnosis were established based on lymph node histologic examination.

Interestingly, we observed an association between high cytoplasmic expression of $\alpha 3\beta 1$ in the ITF and recurrence in OSCC. This association was also previously reported in prostate cancer (Pontes-Junior et al., 2010) and suggests the value of this molecule as a prognostic marker.

Investigation of the correlation of the expression of immunohistochemical markers revealed a significant strong positive correlation between E-cad cytoplasmic expression and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in a primary site in metastatic OSCC. Although interactions among cadherins and integrins have already been noted (Whittard et al., 2002; Chattopadhyay et al., 2003; Zhang et al., 2006; Weber et al., 2011), this is the first investigation to report a correlation between the cytoplasmic expression of E-cad, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in OSCC. This correlation is probably an indication that the loss of intercellular adhesion in cancer cells, due to the abnormal expression of E-cad, is related to increased adhesion to the components of the extracellular matrix mediated by integrins. This statement is supported by the recent findings of Burkhalter et al. in the field of ovarian carcinoma. The

authors demonstrated that the binding of the integrin subunits $\alpha 2$, $\alpha 3$ and $\beta 1$ to collagen induces the redistribution of E-cad expression in the cell, so there is a reduction in expression in the cytoplasmic membrane, and E-cad goes on to be diffusely expressed in the cell cytoplasm. At the same time, the connections between these integrins and the extracellular matrix allow the nuclear translocation of β -catenin and the transcription of target genes of the Wnt canonical pathway. These data demonstrate that cell-matrix interactions mediated by integrins alter the dynamics of the cadherin-catenin complex, thereby increasing cell invasiveness (Burkhalter et al., 2011).

Our study contributes to a better understanding of the role of E-cad, β -cat, and $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in different OSCC microenvironments and it is the first research that provides these expressions in different sites of the lesion as in the LC, ITF and MNC. However, the role of E-cad when expressed in the cytoplasm and nucleus, as well as its relationship with integrins expressed in cytoplasm, aside from their normal expression in the membrane of keratinocytes also requires further investigation.

Future studies that explore the interactions between molecules of adhesion cell-cell and cell-extracellular matrix should be encouraged, especially studies of the cadherin-catenin complex, $\alpha 2\beta 1$, $\alpha 3\beta 1$ and other members of integrins family. Interestingly, it has been shown that other integrins play important role in OSCC and other neoplasms (i.e. $\alpha v\beta 5$, $\alpha 5\beta 1$, and $\alpha 9\beta 1$). These integrins have been associated with invasion, metastasis and chemotherapeutic drug resistance and could represent valuable therapeutic targets (Monnier et al., 2008; Hu et al., 2012; Gupta et al., 2013; Li et al., 2013).

In conclusion, our results suggest that abnormal expression of E-cad and β -cat involving a loss of expression in the cytoplasmic membrane and high expression in the cytoplasm seems to be associated with high expression of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins, which can influence cell motility and survival in metastatic OSCC.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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