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Immunohistochemical evaluation of EGFR expression in lip squamous cell carcinoma. Correlation with clinicopathological characteristics

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Summary. Background: The majority of lip cancer is the squamous cell carcinoma (SCC) type that exhibits clinical and biological characteristics intermediate between skin and oral SCC. The aim of this study was to assess the impact of epidermal growth factor receptor (EGFR) expression on prognosis of lip squamous cell carcinoma (LSCC) and to relate it with clinicopathological features. The role of EGFR expression as a possible therapeutic target was also discussed.

Methods: A series of 55 patients with LSCC was analyzed. EGFR expression was determined by standardized immunohistochemistry (pharmDx assay) and evaluated by both manual and automated image analysis (ACIS III). The Kappa statistic test was used to evaluate the concordance of manual and automated scores. EGFR results were correlated with clinicopathologic characteristics. Statistical differences between proportions were determined by the chi-squared test (with linear-by-linear correction where appropriate). The Mann-Whitney and the Kruskal-Wallis test were employed for comparison of continuous variables.

Results: Correlation between manual and automated score was obtained in 50/55 cases (90.9%). EGFR expression was absent or weak in 14 cases (25.5%); borderline (2+) in 20 cases (36.4%) and positive (3+) in 21 cases (38.2%). Significant relationships were found between EGFR expression and tumour ulceration (p=0.022) and tumour thickness (p=0,002) and width (p=0.021).

Conclusions: Our results revealed EGFR high expression in LSCC and its relationship with bad prognosis criteria (tumour size and ulceration).

Key words: Lip cancer, Squamous cell carcinoma, Immunohistochemistry, EGFR expression, Automated image analysis

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Introduction

Lip cancer is the most common type of orofacial cancer in Caucasian males (Chen et al, 1992). The vast majority of lip cancer is the SCC type and exhibits clinical and biological characteristics intermediate between the less aggressive skin (Dinehart and Pollack, 1989; Rowe et al., 1992) and more aggressive oral SCC (Batista et al., 2010). For this reason, the last version of TNM staging of cancers of the head and neck considers the LSCC as a subtype with special characteristics (Edge et al., 2010; Patel and Shah, 2005).

In recent years the expression of EGFR in different types of cancer has been the focus of great interest because of its relationship with prognosis and its role as a possible therapeutic target. Overexpression of EGFR has been reported in a variety of human cancers, including both oral (Störkel et al., 1993; Chen et al., 2003; Ulanovski et al., 2004; Smid et al., 2006; Diniz-Freitas et al., 2007; Laimer et al., 2007; Agra et al., 2008; Monteiro et al., 2010, 2012; Del Sordo et al., 2010) and nonmelanoma skin cancer (NMSC) (Springer

and Robinson, 1991; Groves et al., 1992; Ahmed et al., 1997; Shimizu et al., 2001; Krähn et al., 2001; Maubec et al., 2005; Fogarty et al., 2007; Ch´ng et al., 2008; Suomela et al 2009). The clinical significance of this expression of EGFR in oral and skin SCC has revealed conflicting results. The study of EGFR expression is of great interest because of the possibility of using this receptor as a target for anticancer therapy with anti-EGFR drugs in SCC of skin, head, and neck (Khalil et al., 2003; Bonner et al., 2006; Bauman et al., 2007; Arnold et al., 2009; Cohen et al., 2009; Griffin et al., 2009; Miller et al., 2010; Bulj et al 2010; Dattatreya and Goswami, 2011; Jedlinski et al., 2013).

To the best of our knowledge, the expression of this receptor in LSCC has not been previously reported in the literature and our aim has been to evaluate EGFR expression in a series of 55 patients with LSCC and to relate it with clinicopathological and epidemiological (smoking and alcohol history) characteristics of the patients.

Materials and methods

Patients

A total of 55 patients, diagnosed with primary LSCC at the University Clinical Hospital of Santiago de Compostela between 1991 and 1999 were included in this study. We have included only the LSCC arising or predominantly located on the vermilion border of the lip. Clinical information (age, gender, smoking and alcohol intake history, primary tumour localization, treatment and evolution) was obtained from the patient files. At the moment of diagnosis, none of them presented distant metastases (exclusion criterion). All patients were followed up until their death or for a minimum of 3 years after treatment.

Materials

Tissue samples were obtained from the files of the Pathology Department of the University Clinical Hospital of Santiago de Compostela. All the tissue samples were reviewed for the following characteristics: tumour size measurement (thickness and width), presence of ulceration, pattern of invasion, and histological differentiation grading. An optical micrometer (Graticules Ltd., Tombridge, UK) was used for tumour depth and width measurement. Tumor thickness (depth) was considered to be the distance from the basal membrane of lip epithelium (or, in ulcerated cases, an imaginary line simulating where the basal membrane would be) to the deepest point of invasion (Gonzalez-Moles et al., 2002). The pattern of invasion was classified into four types (Spiro et al., 1999). Type 1: tumour with consistently well-defined, "pushing" border; type 2: tumour with a pattern of solid cords; type 3: invasion in groups of 15 or more cells; and type 4: invasion in groups of fewer than 15 cells. For data analysis, these categories were subsequently grouped into two broader categories: low invasive (type 1 or 2) and high invasive pattern (type 3 or 4) (Diniz-Freitas et al., 2007). The histopathologic grading was classified into three types: well, moderately, and poorly differentiated, according to the AJCC (Edge et al., 2010) and WHO (Pindborg et al., 1997; Barnes et al., 2005) criteria.

Methods

a) Immunohistochemistry

For immunohistochemical study, 4 micrometer sections were cut, mounted on treated FLEX IHC microscope slides (Dako, Glostrup, Denmark) and heated in an oven at 60°C for one hour. The sections were deparaffinized in xylene, dehydrated in an ethanol series and rinsed in distilled water. All inmunohistochemistry procedures were performed using an AutostainerLink 48 (Dako).

For EGFR immunostaining, the EGFR pharmDx kit (Dako) was used, following the manufacturer's instructions. Cell line controls provided with the kit were used in each staining run. Normal epithelium was tested as positive internal control (not shown).

b) Evaluation of EGFR expression

Manual IHC scoring. Only plasma membrane staining was considered positive. EGFR expression was evaluated on the basis of extent and intensity of EGFR immunolabeling in tumour cell membranes, classified on a four-point scale following the instructions provided with the kit: 0 (no labeling, or labeling in <10% of tumour cells); 1+ (weak labeling, homogeneous or patchy, in >10% of tumour cells); 2+ (moderate labeling, homogeneous or patchy, in >10% of tumour cells); 3+ (intense labeling, homogeneous or patchy, in >10% of tumour cells). All samples were simultaneously evaluated by four authors (AC, MG, MD and TG-C), and the final manual scoring was determined by consensus.

Automated IHC scoring. For automated scoring the ACIS III (Automated Cellular Imaging Systems, Dako, Carpinteria, California) was used. The ACIS III system scans the slide and is capable of differentiating membrane from cytoplasmic staining. Five representative areas were selected by an author (IF-C) who was unaware of the manual results and the system generated an average score. For EGFR evaluation we used the ACIS III HER2 scoring system: scores below 1.5 were considered negative (0/1+); scores between 1.5 and 2.5 were considered borderline (2+) and scores of 2.5 or higher were considered positive (3+) for EGFR. The manual and automatic scores were compared and, in case of discrepancy, the automatic score was considered as the definitive result. For statistical analysis, these

categories were subsequently grouped into two wider categories: negative or borderline (0/1+/2+) and positive (3+).

c) Statistical analysis

Continuous variables are given as mean ± SD. Statistically significant differences between proportions were determined by the chi-squared test (with linear-by-linear correction where appropriate). The Mann-Whitney and the Kruskal-Wallis test were employed for comparison of continuous variables. The Kappa statistic test was used to evaluate the concordance of manual and automated scores. P<0.05 was considered to indicate a statistically significant difference.

Results

We studied several epidemiological, clinical, and histopathological parameters regarding our series of 55 patients with LSCC (Table 1).

The age of patients ranged from 23 to 96 years (mean 74.0±14.6) and the ratio male:female was 4:1. Ninety-one per cent of LSCC were located on lower lip. The mean thickness was 5.8±3.6 mm (range, 1.5-18.3 mm), so all patients were in T1 category of TNM classification. The mean tumour width was 10.9±4.8 mm (range, 0.9-22.2 mm). Eighty per cent of tumours were well differentiated and only 1 case was poorly differentiated. Only two cases presented vascular and/or perineural invasion.

Twelve out of 50 patients (24%) had local recurrences, and 4 (8%) had lymph node metastasis. The median time lapse for local recurrences was 6.5 months (range: 2.9-121.4). The appearance of local recurrences was not related to age, sex, tumour size, ulceration, or EGFR expression. Lymph node metastasis was observed in 4 patients and 3 of them showed strong positivity (3+) for EGFR. Only one patient in our series developed distant metastasis (lung) and, strikingly, his was the only tumour that was poorly differentiated, and strongly expressed EGFR (3+). In addition, this patient had local recurrences and lymph node metastasis within 3 years of initial LSCC diagnosis.

Seven patients (12.7%) from our series developed other NMSC on the sun-exposed skin and 13 patients (23.6%) died from other malignancies.

EGFR expression

EGFR immunostaining was localized in the plasma membrane, although positivity was also observed in the cytoplasm in cases with intense plasma membrane immunostaining (3+). Cell line controls performed in each staining run confirmed the sensitivity and specificity of the assays.

Using manual scoring for EGFR, 8 tumours were 0 (14.5%); 8 were 1+ (14.5%); 19 were 2+ (34.5%); and 20 were 3+ (36.4%). Using automate scoring, EGFR was negative (0/1+) in 14 tumours (25.5%); borderline (2+) in 20 cases (36.4%) and positive (3+) in 21 cases (38.2%). Examples of different levels of EGFR expression are given in Fig. 1. Concordance between manual and automated image analysis (ACIS III) score

Table 1. Clinicopathological features and EGFR expression.

	Variable	N (%)	EGFR expression		p
			Negative/Bordeline (N=34, 61,8%)	Intense (N=21, 38,2%)	
Age	< 75 years	28 (50.9)	19 (55.9)	9 (42.9)	0.348
	>75 years	27 (49.1)	15 (44.1)	12 (57.1)	
Gender	male	44 (80.0)	26 (76.5)	18 (85.7)	0.405
	female	11 (20.0)	8 (23.5)	3 (14.3)	
Tobacco habit (1)	no smoking	18 (37.5)	10 (34.5)	8 (42.1)	0.594
	smokers	30 (62.5)	19 (65.5)	11 (57.9)	
Alcohol intake (2)	< 40 g/day	19 (40.4)	14 (50.0)	5 (26.3)	0.104
	> 40 g/day	28 (59.6)	14 (50.0)	14 (73.7)	
Ulceration	absent	18 (32.7)	15 (44.1)	3 (14.3)	0.022 (*)
	present	37 (67.3)	19 (55.9)	18 (85.7)	
Invasion pattern	low grade	24 (43.6)	16 (47.1)	8 (38.1)	0.515
	high grade	31 (56.4)	18 (52.9)	13 (61.9)	
Histological differentiation	well	44 (80.0)	26 (76.5)	18 (85.7)	0.405
	moderate/poor	11 (20.0)	8 (23.5)	3 (14.3)	
Lymph node metastasis (3)	absent	46 (92.0)	29 (96.7)	17 (85.0)	0.289
	present	4 (8.0)	1 (3.3)	3 (15.0)	
Local recurrences (4)	absent	38 (76.0)	22 (73.3)	16 (80.0)	0.693
	present	12 (24.0)	8 (26.7)	4 (20.0)	

Values in parentheses are percentages. Data not available in 7 (1), 8 (2), 5 (3, 4) clinical records. (*) Statistically significant (p<0.05)

was obtained in 50/55 cases (90.9%) with an excellent concordance (k=0.86) (Landis and Koch, 1977) (Table 2). Cases with scoring discrepancies were always near the ACIS III threshold. In 4/5 cases the manual score was lower than the automated one (in two cases the manual result was 0/1+ and the automated one was 2+, and in another two cases the manual result was 2+ and the automated one was 3+). In one case the manual score was higher (3+) than the automated one (2+). In the case of discrepancy, the automated score was considered to be definitive. Grouping the categories together for statistical analysis, EGFR expression was negative/borderline in 34 cases (61.8%) and intense in 21 cases (38.2%).

Table 2. Crosstabulation of EGFR result for automated and manual analysis.

		EGFR result Automated image analysis (ACIS III)			Total
		0/1+	2+	3+	
EGFR result Manual analysis	0/1+	14	2	0	16
	2+	0	17	2	19
	3+	0	1	19	20
Total		14	20	21	55

Agreement: 90.9%. Kappa Value: 0.86 (almost perfect agreement)

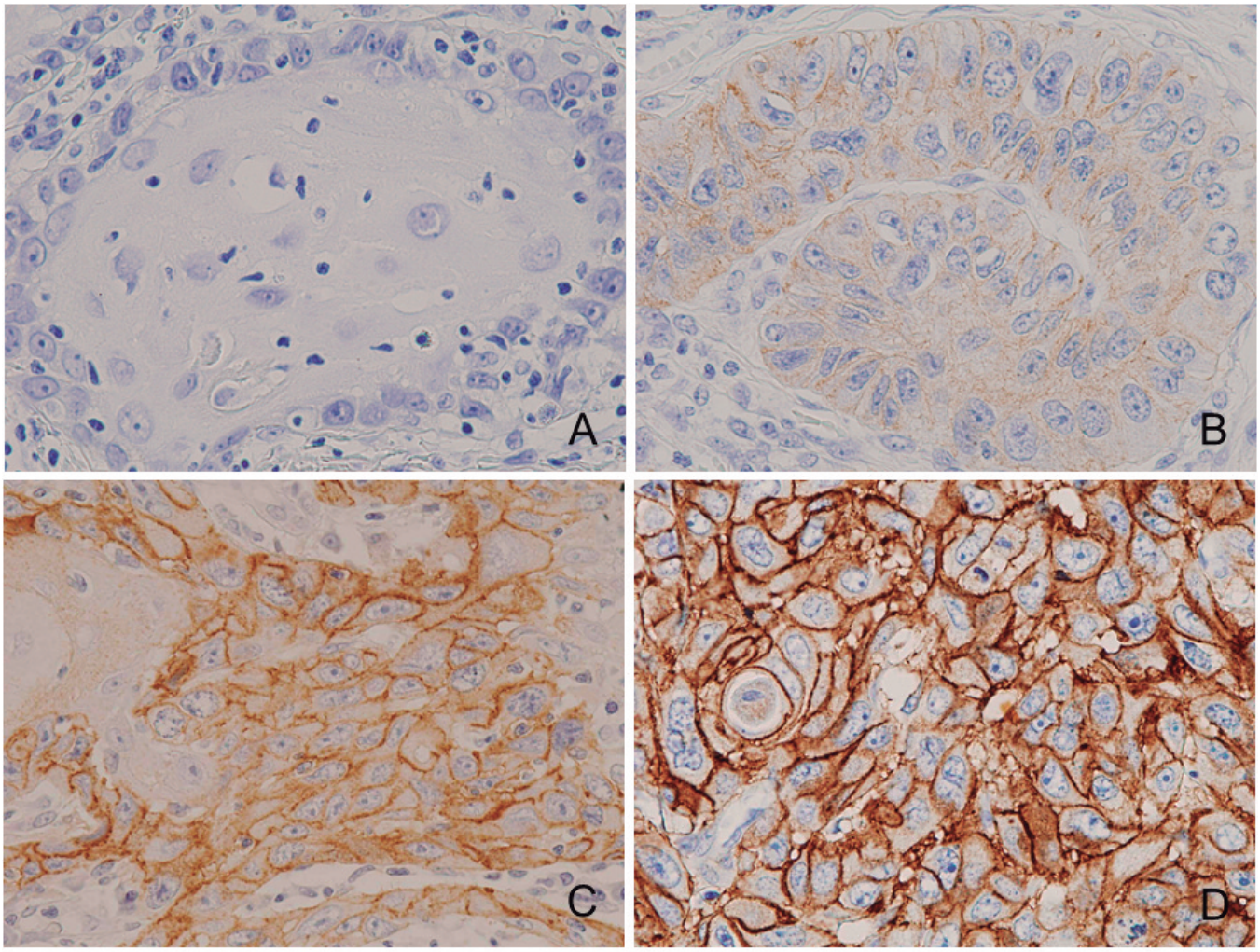

Fig. 1. Immunohistochemical staining of EGFR in lip squamous cell carcinomas. Examples of the different scores. **A**. 0: No immunostaining was found in this case. **B.** 1+: Weak and incomplete membrane labeling is seen in approximately a half of the tumour cells. **C.** 2+: Most tumour cells showed moderate and complete membrane immunoreactivity. **D.** 3+: Intense and complete membrane positivity was observed in virtually all tumour cells (weak to moderate cytoplasmic staining was also seen in occasional cells). x 40

Correlation between EGFR and clinicopathological features

EGFR expression was analysed in relation to clinicopathological variables (Table 1). An association was found between EGFR expression and tumour ulceration (p=0.022) and tumour thickness (p=0.002) and width (p=0.021). The mean thickness was 7.7±3.9mm in tumours with 3+ EGFR expression versus 4.7±3.0mm in tumours with negative/borderline EGFR expression (p=0.002). In tumours expressing EGFR intensely the mean width was 12.8±4.6 mm, while in negative/borderline cases it was 9.7±4.7mm (p=0.021). The rest of the variables studied showed no significant correlations.

Discussion

Classically, lip cancer has been included in the group of head and neck cancers. This encompasses a broad and heterogeneous group of malignancies with diverse clinical behavior and outcomes depending on anatomic sites from which tumours can arise and the diversity of histological tumour types. There is also considerable confusion in the literature because some authors include lip cancer within the oral cancer group, while others consider lip cancer to be a separate entity encompassing all the malignancies located on skin, vermilion, and mucous membranes of the lip (Czerninski et al., 2010). The lip vermilion is a special transitional zone between the glabrous skin and the mucous membrane of oral cavity with distinctive histopathological peculiarities (semimucosal tissue) and it is reasonable to think that the malignances arising on this area will be different from skin and oral cancers. The vast majority of lip cancer is SCC (Chen et al., 1992; Perea-Milla et al., 2003; Maruccia et al., 2012). In fact, the LSCC is considered to be a subtype of head and neck cancer with special characteristics because its clinical and biological behavior is intermediate between skin and oral SCC (Chen et al., 1992; Patel and Shah, 2005; Nguyen and Yoon, 2005; Edge et al., 2010). There are also epidemiological reasons that support the lip as a distinct cancer site (Chen et al., 1992; Moore et al., 1999; Czerninski et al., 2010). For these reasons, the last version of TNM staging of cancers of the head and neck considers LSCC to be an independent subtype of cancer (Patel and Shah, 2005; Edge et al., 2010). This study has only considered SCC arising in lip vermilion to be LSCC.

Although EGFR expression in oral and skin SCC has been studied, to our knowledge the expression of this receptor in LSCC has not been previously reported. The aim of this study was to evaluate EGFR expression in a series of patients with LSCC and to relate it with their clinicopathological characteristics.

We have studied a series of 55 patients with LSCC. In general, the epidemiologic, clinical, and histopathological findings from our series were similar to those reported previously. The mean age of 74 years in our series is consistent with prior reports. The male:female ratio of 4:1 is the same as reported by other authors (Chen et al., 1992; Maruccia et al 2012). Ninety-one percent of our patients had the LSCC located on the lower lip, and this is in accordance with the findings by Maruccia et al. (2012), Chen et al. (1992), Antoniades et al. (1995) and Perea-Milla Lopez et al. (2003), who reported the same location in 79, 85, 93 and 100% of their patients, respectively. 62.5% of our patients were smokers and 44.7% drank more than 40 gr of ethanol daily, which is in line with previous reports (Maruccia et al., 2012). Although tobacco and alcohol are classically accepted as etiologic factors for LSCC, we think the main etiological factor is actually chronic exposure to sun (ultraviolet radiation). This is supported by the fact that the majority of LSCC are located on the photoexposed portion of the vermilion of lower lip while they are rare on upper lip (with less ultraviolet exposure) (Chen et al., 1992; Antoniades et al., 1995; Perea-Milla Lopez et al., 2003; Czerninski et al., 2010; Maruccia et al., 2012). Other epidemiologic evidence is the lower incidence of LSCC in dark-skinned populations (Moore et al., 1999). Although with less impact than ultraviolet radiation, tobacco and alcohol have been implicated in the pathogenesis of LSCC (Chen et al., 1992; Perea-Milla Lopez et al., 2003; Maruccia et al., 2012), but their true value is debated (Moore et al., 1999), especially for alcohol because of a potential confounding with tobacco.

Twenty-eight percent of our patients had local recurrences and 7.3% had lymph node metastasis, while only one patient developed distant metastasis (lung). We must point out that it is very difficult (even impossible) to distinguish recurrence from a second primary tumour because both main carcinogens (ultraviolet radiation and tobacco) have a diffuse effect on the lip (clinically expressed as actinic cheilitis and/or leukoplakia nearby or adjacent to the tumour). This multifocal origin of cancer has also been described in other parts of the digestive tract.

Four patients (7.3%) had lymph node metastasis which appeared in relation to ulceration and expression of EGFR (see Table 1). Other authors have reported a similar result, i.e., 7 % of lymph node metastasis (Antoniades et al., 1995). It is remarkable that only lymph node metastasis and not local “recurrences” were related to other parameters of poor prognosis (ulceration and EGFR expression), which supports the fact that recurrences are indistinguishable from a second primary tumour. Less frequent was the appearance of distant metastasis, which occurred in only one patient in our series. This patient had the only LSCC classified as poorly differentiated and previously presented local recurrences and lymph node metastasis. This tumour also expressed EGFR (3+) strongly.

In our series the global 5-year survival rate was 68%, which is in accordance with previous reports of rates over 80% (Antoniades et al., 1995; Moore et al., 1999). We share the opinion of other authors that this

good overall prognosis depends more on the easy diagnosis and effective treatment of LSCC than on the biological behavior of this malignancy (Moore et al., 1999).

The grade of histological differentiation of the primary tumour has a radical influence on 5-year survival rate (Antoniades et al., 1995). In our series, only one tumour (1.8%) was poorly differentiated, while 10 were moderately differentiated (18.2%) and 44 were well differentiated (80.0%). Chen et al (1992) in their series of 2291 patients reported 7%, 48%, and 44% of poorly, moderately, and well differentiated tumours. Antoniades et al. (1995) reported that 7, 29 and 64% of 408 evaluated LSCC were poorly, moderately, and well differentiated. The low number of cases in our series could explain the differences. In any case, only a minority of LSCC were poorly differentiated, which could explain the low global aggressiveness of this malignancy.

EGFR plays a crucial role in autocrine stimulation of cancer extension, including growth, differentiation, inhibition of apoptosis, and metastatic progression (O-Charoenrat et al., 2002). Overexpression of EGFR has been reported in a variety of human cancers, including both oral (Störkel et al., 1993; Chen et al., 2003; Ulanovski et al., 2004; Smid et al., 2006; Diniz-Freitas et al., 2007; Laimer et al., 2007; Agra et al., 2008; Monteiro et al., 2010, 2012; Del Sordo et al., 2010) and NMSC (Springer and Robinson, 1991; Groves et al., 1992; Ahmed et al., 1997; Shimizu et al., 2001; Krähn et al., 2001; Maubec et al., 2005; Fogarty et al., 2007; Ch´ng et al., 2008; Suomela et al 2009). EGFR is implicated in the pathogenesis of NMSC (El-Abaseri et al., 2006; Rittie et al., 2007; Schneider et al., 2008) and this role is also supported by the fact that this receptor is activated by ultraviolet radiation (El-Abaseri et al., 2006; Singh et al., 2009). This role seems more marked on SCC that in basal cell carcinoma (Ahmed et al., 1997; Rittie et al., 2007). The clinical significance of EGFR overexpression in NMSC and oral cancer is unclear. Some reports suggest that the overexpression of EGFR is not related to prognosis and/or biological behavior of the tumour, either in oral (Ulanovski et al., 2004; Smid et al., 2006; Diniz-Freitas et al., 2007; Monteiro et al., 2010; Agra et al., 2008) and skin SCC (Fogarty et al., 2007). Nevertheless, other authors have found that overexpression of EGFR has a prognostic value in oral (Chen et al., 2003; Laimer et al., 2007; Del Sordo et al., 2010; Monteiro et al., 2012) and skin SCC (Shimizu et al., 2001). Overexpression of EGFR in primary skin SCC is significantly associated with the development of subsequent metastasis (Ch´ng et al., 2008) and this receptor is also overexpressed in metastatic skin SCC (Shimizu et al., 2001; Maubec et al., 2005).

The study of EGFR expression is of great interest in the treatment of EGFR-expressing malignancies because of the new therapeutic possibilities involving the administration of specific EGFR antibodies (cetuximab) or tyrosine kinase inhibitors (erlotinib and gefitinib) (Khalil et al., 2003), including also when combined with radiotherapy (Pedicini et al., 2012a,b).

In fact, recent case-reports suggest that the use of biological anti-EGFR drugs is effective in the treatment of advanced skin SCC (Bauman et al., 2007; Arnold et al., 2009; Miller et al., 2010; Bulj et al., 2010) and other SCC of the head and neck (Bonner et al., 2006; Cohen et al., 2009; Griffin et al., 2009; Dattatreya and Goswami, 2011). In head and neck SCC cell lines, the overexpression of EGFR was correlated with cetuximab sensitivity (Jedlinski et al., 2013).

To the best of our knowledge, there are no prior reports in the literature about the expression of EGFR limited to LSCC. In this study, we have evaluated EGFR immunostaining by manual and automated methods. Ciampa et al. (2006) found that automated scoring with ACIS system reduced interobserver variability and increased the correlation of IHC and FISH (93% vs. 71% by manual IHC scoring) in HER2 evaluation. We obtained a concordance of 91% between manual and automated image analysis score and the same result was reported for HER2 in breast cancer by other authors (Dobson et al., 2010). EGFR expression in LSCC was absent or weak in 26% of cases; borderline (2+) in 36% and positive (3+) in 38.2%. Similar results (25%, 41% and 34%, respectively) were previously reported by our group in oral SCC (Diniz-Freitas et al., 2007).

We have found that EGFR expression in LSCC is related to two clinicopathological variables of negative prognosis: tumour size and ulceration. This finding is in accordance with other reports that consider EGFR expression to be an unfavourable factor both in oral (Chen et al., 2003; Laimer et al., 2007; Del Sordo et al., 2010; Monteiro et al., 2012) and skin (Shimizu et al., 2001; Maubec et al., 2005; Ch´ng et al., 2008) cancers. Nevertheless, this observation must be considered with caution because most patients with high EGFR expression died from causes other than LSCC. This could be explained by the fact that LSCC is usually diagnosed in early stages due to visible location, and that surgical treatment is usually curative independently of other bad-prognosis factors (tumour size, ulceration, histological grade, EGFR expression) (Antoniades et al., 1995; Moore et al., 1999).

In summary, the epidemiologic, clinical, and histopathological findings in our series of LSCC demonstrate higher expression of EGFR that correlates with ulceration and tumour size and might be associated with bad prognosis.

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