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ORIGINAL ARTICLE

Correlation between Skp2 expression and nodal metastasis in Stage I and II oral squamous cell carcinomas

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Purpose: The aim of this study was to investigate the role of S-phase kinase associated protein (Skp2) in the development of nodal metastasis and to assess its influence on prognosis in stage I and II oral squamous cell carcinomas (OSCCs).

Experimental Design: Seventy-one patients affected by OSCC (stage I-II) were observed in the period ranging from March 2003 to December 2006. The research was performed using immunohistochemical and histopathological analysis.

Results: The overall survival rate was 89.6% at 3 years, 87% at 5 years and 80.7% at 10 years. Patients with vascular or perineural invasion showed no statistically significant survival difference when compared with the ones with no invasion. The tumour depth of invasion did not prove to be related to the metastatic potential.

Nine of the seventeen patients with Skp2 positive nuclei (≥20%) developed nodal metastasis. Conversely, only 6 of the 54 patients with a nuclear positivity lower than 20% developed a laterocervical metastasis ($P = 0.001$). When comparing survival curves of Skp ≥ 20% and Skp2 < 20% OSCCs, no significant P value emerged from the statistical analysis.

Conclusions: This study is the first to report an important correlation between an Skp2 expression lower than 20% and the capability of the tumour not to develop nodal laterocervical metastases ($P = 0.001$).

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Keywords: Skp2; p27; oral carcinoma; nodal metastasis

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Statement of Translational Relevance

The present study is the first to report an important correlation between an Skp2 expression lower than 20% and the capability of the tumour not to develop nodal laterocervical metastases ($P = 0.001$). Even though further research is needed, the data presented in this study could prove to be the basis for developing new criteria which may help surgeons to address the issue of laterocervical dissection in Stage I and II OSCCs.

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Introduction

Nodal metastases represent the most important prognostic factor in the evaluation of oral squamous cell carcinomas (OSCCs): the 5-year survival rate decreases down to 50% in node-positive patients (N+), together with an increased risk of developing distant metastases (Regezi and Sciubba, 1989). Woolgar reports a 5-year survival equal to 81% for N0 (according to TNM staging system) patients, 64% for patients presenting intranodal metastases and 21% for those with nodal metastasis and extra-capsular diffusion (Woolgar *et al*, 1999).

Despite this, a unanimous consensus on laterocervical nodal treatment in stage I and II OSCC patients has not been reached yet. The literature presents diverse attempts at establishing factors related to probability of metastasis (perineural invasion, vascular invasion, inflammatory exudate, grading, pattern and depth of invasion) as an aid for the clinician when addressing the choice of whether a laterocervical dissection should be carried out in early stages of OSCCs (Garzino-Demo *et al*, 2006).

In addition to these factors, several tissue and biological markers have been taken into consideration as possible indicators of tumour aggressiveness and predictors of the carcinogenic process and of the metastatic capability of the tumour itself (Brinkman and Wong, 2006).

The S-phase kinase associated protein (Skp2) belongs to the F-box protein family (Fbps) and is able to break down p27 through ubiquitination. It also favours the transition from the G1 to the S phase of the cell cycle (Pagano *et al*, 1995; Fredersdorf *et al*,1997; Loda *et al*, 1997; Porter *et al*,1997; Carrano *et al*, 1999; Tsvetkov *et al*, 1999). Furthermore, the forced expression of Skp2 in quiescent fibroblasts triggers DNA synthesis (Sutterlüty *et al*, 1999). An Skp2 overexpression has been noticed in diverse tumour types and is indicative of poor prognosis in patients affected by OSCC, gastric cancer,

colon, ovarian, Gastrointestinal tract, pulmonary cancers and lymphomas (Woolgar and Scott, 1995; Hershko *et al*, 2001; Kudo *et al*, 2001; Latres *et al*, 2001; Harada *et al*, 2005a,b). These observations lead to the thought that Skp2 might have oncogenic properties.

This work aims at identifying the relationship between both ipsilateral laterocervical and distant metastases in OSCCs and the semi-quantitative expression of Skp2 in cells found at the invasive tumour front in clinical and instrumental (TC and MR) N0 patients. Moreover, the correlation between overall survival of the examined sample and Skp2 expression was studied. The depth of tumour invasion (2 mm cut-off) was evaluated in relation to the metastatic ability of the tumour.

Materials and methods

Seventy-one patients affected by OSCC (stage I-II) came to our attention (Department of Maxillo-Facial Surgery, University of Turin, Italy) and were involved into this study (Table 1). Forty-one of these (57.7%) were males and 30 females (42.3%). The mean age of subjects was 62 (range 24–82).

Thirty-five of the 71 patients (49.3%) presented lesions localised to the buccal mucosa, whereas in 36 cases (50.7%) the tongue margin and/or floor of the mouth were involved.

Research was carried out onto the definitive anatomopathological findings (surgical pieces) which were evaluated through both a histopathological assessment (depth of invasion) and immunohistochemistry. The latter made use of a semiquantitative method (protein Skp-2 expression).

The tumour depth of invasion (2 mm cut-off) and its relationship with the presence of a laterocervical nodal metastasis and/or a distant metastasis was also assessed.

Data were then correlated with the presence of ipsilateral nodal metastasis, distant metastasis and with the cumulative survival of the examined sample with a minimum 3-year follow-up.

Immunohistochemistry

The immunohistochemical evaluation was carried out onto 4-micron thick specimens, each obtained by fixation of the pieces into 4% formalin and subsequent paraffin inclusion. Sections were then deparaffined in xylol and hydrated through the passage in a decreasing alcohol series. The activity of endogenous peroxidases was subsequently inhibited by using 0.3% H_2O_2 for 15 min. To intensify the signal, antigen demasking was carried out by means of two microwave cycles (15 min each), adopting a citrate buffer (pH 6.2). The monoclonal Mouse anti-Skp2 antibody (clone 2C8D9, *Zymed*) was adopted to assess the expression of Skp2. Sections were then incubated with the above-mentioned antibody at 1:100 dilution for 60 min at room temperature. The avidin-biotin-peroxidase complex, which uses diaminobenzidine (DAB) as chromogene, was adopted. After the process of oxidation is completed, DAB precipitates where the antigen-antibody bond has taken place. Normal mouse serum containing immunoglobulins at a concentration similar to that of the primary antibody was adopted as a negative control.

Immunostained sections were then evaluated by three independent observers. None of them had any knowledge of the clinical/histological diagnosis. For each examined specimen section, the mean percentage of cells which resulted to be positive at the invasive front was evaluated. The percentage of positively-stained cells in each specimen was estimated by counting the number of cells per area from at least three different areas. The nuclear positivity for the antibody was considered to be specific. On the basis of previous works found in the literature, this study took into account a cut-off value of 20% to sub-divide samples into those with a high and those with a low Skp2 protein expression (Figures 1 and 2) (Gstaiger *et al*, 2001; Hershko *et al*, 2001; Harada *et al*, 2005a,b).

Statistical analysis

The statistical analysis was carried out by means of Kaplan–Meier survival curves and considering overall survival, stage and Skp2 expression separately. The statistical significance was assessed with log-rank tests.

Chi-square and Fisher's exact tests were adopted to carry out the univaried analysis of metastatic capability in relation with both Skp2 expression and depth of invasion.

The day of surgery was considered as time zero. Patients who remained alive were included in the group of 'subjects at risk of death due to disease' up to the last follow-up. This is why survival curves change only when the subject deceases.

Survival curves have been estimated to be realistically compatible with the actual survival curves. The statistical analysis was carried out using the STATA 8 software Science Plus Group, Groningen, Netherlands.

Results

The overall patient survival was evaluated by means of Kaplan–Meier algorithms, which revealed 3, 5 and 10-year overall rates equal to 89.6%, 87% and 80.7%, respectively.

When analysing survival in relation to tumour stage, the results showed a non-statistically significant difference with a Chi square value of 0.69 and $P = 0.42$ (Figure 3).

Correlation between tumour depth of invasion and both nodal and distant metastases

Fifty-two of the 71 patients presented a depth of invasion ≥2 mm. Of these, 13 developed a metastasis. The results reported in Figure 1 are not correlated in a statistically significant fashion ($P = 0.186$).

Correlation between Skp2 expression and both nodal and distant metastases

Seventeen of the 71 patients considered in this study showed Skp2 positive nuclei (≥20%). Of these seventeen, nine developed a laterocervical metastasis in time. Conversely, only 6 of the 54 patients with a nuclear positivity lower than 20% developed a laterocervical metastasis (Table 2).

Table 1 Patients included in this study and their tumour stage, depth of invasion, follow-up, Skp2 expression and 5-year survival

Patient	*Stage*	*Depth of invasion (mm)*	*Follow-up*	*Skp2 expression*	*Survival (5 years)*
V.P.	I	<2	Free of disease	10%	Alive
M.I.	I	>2	Laterocervical metastasis	25%	Alive
L.E.	I	>2	Free of disease	5%	Alive
P.A.	I	>2	Free of disease	1%	Deceased
B.M.R.	I	<2	Free of disease	Neg.	Alive
P.P.	I	>2	Free of disease	5%	Deceased
C.R.	I	<2	Free of disease	5%	Alive
A.R.	I	>2	Free of disease	5%	Alive
M.G.	I	<2	Free of disease	10%	Deceased
S.P.	I	<2	Free of disease	Neg.	Alive
M.E.	I	>2	Free of disease	Neg.	Alive
P.E.	I	>2	Laterocervical metastasis	15%	Deceased
L.F.	I	>2	Local recurrence	10%	Alive
R.I.	I	>2	Laterocervical metastasis	20%	Alive
C.G.	II	>2	Free of disease	5%	Alive
S.M.	II	>2	Free of disease	10%	Alive
K.M.	I	<2	Laterocervical metastasis	30%	Alive
G.G.C.	I	>2	Free of disease	10%	Deceased
G.L.	I	>2	Free of disease	10%	Alive
M.C.	I	<2	Free of disease	10%	Alive
V.F.	I	>2	Free of disease	Neg	Alive
M.A.	II	>2	Free of disease	2%	Alive
N.V.	I	>2	Free of disease	10%	Alive
G.R.	I	>2	Free of disease	10%	Alive
V.C.L.	II	>2	Free of disease	25%	Alive
R.R.	II	>2	Laterocervical metastasis	20%	Deceased
R.M.	II	>2	Local recurrence	10%	Deceased
C.A.	II	>2	Free of disease	30%	Alive
A.G.	I	>2	Laterocervical metastasis	10%	Alive
Z.M.	I	<2	Free of disease	5%	Alive
F.L.	I	<2	Free of disease	5%	Alive
M.I.	I	<2	Free of disease	10%	Alive
A.G.	I	>2	Laterocervical metastasis	25%	Alive
C.S.	I	>2	Free of disease	15%	Alive
G.M.	II	>2	Free of disease	5%	Alive
B.L.	I	<2	Local recurrence	5%	Deceased
M.Z.A.	I	>2	Free of disease	5%	Alive
G.G.	II	>2	Laterocervical metastasis	Neg	Alive
B.S.	I	>2	Free of disease	Neg	Alive
S.M.	II	>2	Free of disease	5%	Alive
P.F.	II	>2	Laterocervical metastasis	10%	Alive
S.B.	I	>2	Free of disease	40%	Alive
P.G.	I	<2	Free of disease	Neg.	Alive
R.A.M.	II	>2	Laterocervical metastasis	25%	Alive
L.B.G.	II	>2	Free of disease	Neg	Alive
F.S.	II	>2	Free of disease	Neg	Alive
D.A.	I	>2	Laterocervical metastasis	30%	Alive
E.S.	II	>2	Free of disease	15%	Alive
V.G.	II	>2	Free of disease	15%	Alive
P.P.	I	<2	Laterocervical metastasis	10%	Alive
D.R.F.	II	>2	Free of disease	neg	Alive
G.T.	II	>2	Laterocervical metastasis	neg	Deceased
G.F.	I	<2	Free of disease	5%	Alive
B.M.	I	>2	Free of disease	15%	Alive
M.C.	II	>2	Lung metastasis	30%	Alive
L.G.	I	>2	Free of disease	10%	Alive
A.P.A.	I	<2	Free of disease	25%	Alive
R.L.	II	>2	Free of disease	5%	Alive
S.F.	I	>2	Free of disease	2%	Alive
P.S.	I	>2	Free of disease	15%	Alive
C.A.	I	<2	Free of disease	10%	Alive
M.N.	I	>2	Free of disease	20%	Alive
C.P.	II	>2	Free of disease	2%	Deceased (other cause)
B.G.	I	<2	Free of disease	5%	Alive
P.A.	II	>2	Free of disease	15%	Alive
G.T.T.	I	>2	Local recurrence	20%	Deceased
C.M.	II	>2	Local recurrence	40%	Deceased
A.C.	II	>2	Laterocervical metastasis	20%	Deceased
R.G.	I	<2	Free of disease	10%	Alive
F.L.	I	>2	Free of disease	30%	Alive
P.C.	I	<2	Free of disease	10%	Alive

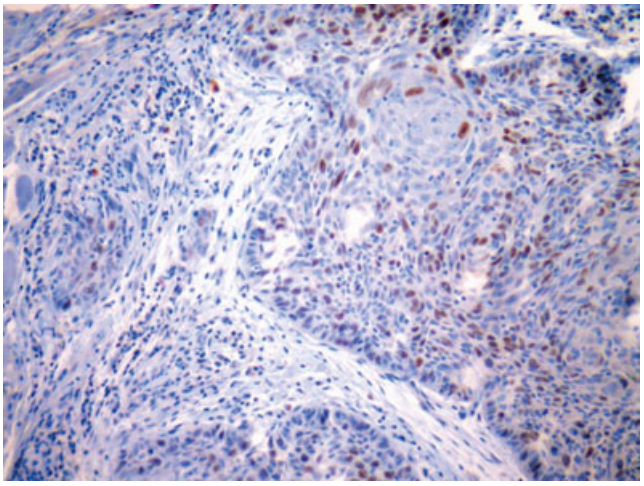

Figure 1 Specimen showing high Skp2 positivity (≥20% stained nuclei)

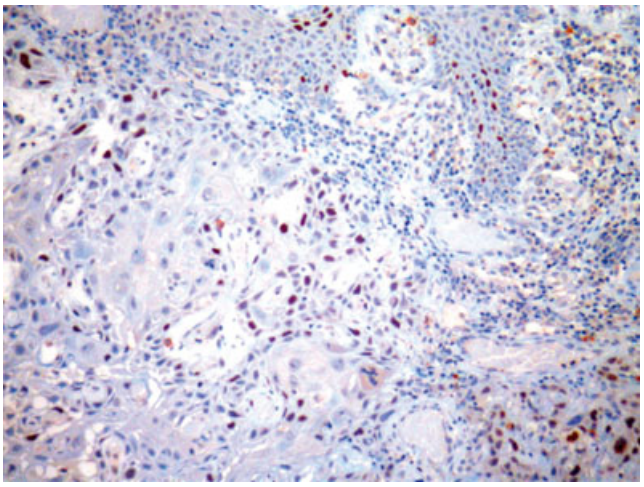

Figure 2 Specimen showing low Skp2 positivity (<20% stained nuclei)

Table 2 Correlation between tumour depth of invasion and both nodal and distant metastases

	< 2 mm	*≥2 mm*	*Total*
No Metastasis	17	39	56
Metastasis	2	13	15
Total	19	52	71

Table 3 Correlation between Skp2 expression and both nodal and distant metastases

	skp2 < 20%	*skp2 ≥ 20%*	*Total*
No metastasis	48	8	56
Metastasis	6	9	15
Total	54	17	71

Only one Skp2-positive patient eventually resulted to be affected by a distant metastasis (lung).

Data shown in Table 3 were evaluated by means of Chi-square test, obtaining a value of 13.5768. The probability was equal to 0.000. Fisher's exact test was carried out for this value with $P = 0.001$.

Correlation between Skp2 expression and survival curves

The statistical analysis carried out by means of survival curves highlighted a non-significant *P* value. Nevertheless, all deaths in patients who presented Skp2 values ≥20% took place within 3 years from surgery. In contrast with this, deaths in subjects with Skp2 < 20% were observed to be equally distributed over a 9-year time lapse.

One patient only died as a consequence of unrelated circumstances, whereas 12 patients died because of disease (Figure 4). Of these 12, four patients showed an Skp2 positivity≥20% and the remaining eight subjects presented Skp2 values <20%.Such difference proved not to be statistically significant with Chi-Square = 0.87 and $P = 0.35$.

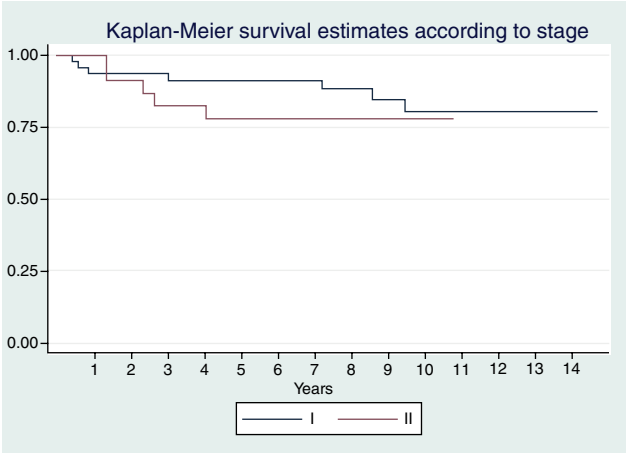

Figure 3 Survival curves related to tumour stage. This analysis shows no statistically significant difference in survival when comparing Stage I and Stage II tumours

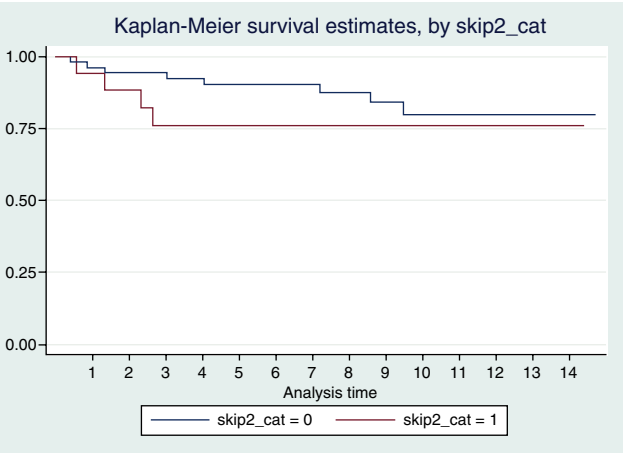

Figure 4 Survival curves in relation to Skp2 expression. This analysis shows no statistically significant difference in survival rates between patients showing Skp2 ≥ 20% and those showing Skp2 < 20%

Discussion

Depth of invasion is believed to be one of the most important prognostic factors in early stages of oral squamous cell carcinoma (Woolgar and Scott, 1995; Po Wing Yuen *et al*, 2002; O-Charoenrat *et al*, 2003; Alkureishi *et al*, 2008; Shah and Gil, 2009). A recent review of the literature (Kudo *et al*, 2005) pointed out that when considering depth of invasion, the cut-off value adopted by different studies ranges from 1.5 mm (Mohit-Tabatabai *et al*,1986) to 10 mm (Al Rajhi *et al*, 2000). At present, there is neither agreed nor unambiguous cut-off value yet (Pentenero *et al*, 2005; O'Brien *et al*, 2003). This study adopted a 2 mm cutoff, the rationale behind this choice being that of distinguishing the metastatic potential of a microinvasive versus an invasive carcinoma (Crissman and Zarbo, 1989).

Of the 71 patients who took part in the study, 52 showed a depth of invasion >2 mm. Thirteen of these developed a laterocervical metastasis and such a correlation resulted not to be statistically significant ($P = 0.186$).

However, the present work aimed at focusing on Skp2 analysis mainly. When considering oral squamous cell carcinomas, as a result of the emerging need to improve therapeutic strategies in early stages (T1-T2), the identification of a biomarker whose expression might be correlated to prognosis and metastatic potential of the tumour itself becomes increasingly important. The determination of such a marker by means of immunohistochemistry would be the most convenient and valid method of risk stratification of patients.

The applications of the oncoprotein Skp2 have recently become of remarkable interest when evaluating prognosis in diverse human carcinomas (Hershko, 2008). Its overexpression has been observed in different malignant tumours, including OSCCs (Gstaiger *et al*, 2001; Hershko *et al*, 2001; Kudo *et al*, 2001; Latres *et al*, 2001; Masuda *et al*, 2002), although the mechanism that leads to such an overexpression remains unknown (Lin *et al*, 2006). Skp2 belongs to the F box protein family and it performs its oncogenic activity by modulating the ubiquitination and degradation of p27 (Pagano *et al*,1995; Fredersdorf *et al*,1997; Loda *et al*,1997; Porter *et al*,1997; Carrano *et al*,1999; Tsvetkov *et al*,1999). The conversion of healthy cells into tumoural cells takes place through a multi-step process that is associated with an accumulation of genetic modifications which involves also oncogenes and tumour suppressor genes (Marx,1989). Some suggest that proliferation and the subsequent tumour progression might actually be caused by anomalies of diverse positive and negative cell cycle regulators (Hunter and Pines, 1994; Sherr, 1996). Progression of the cell cycle is positively regulated by multiple cyclins and cyclin-dependent kinases (CDKs). The cyclin/CDK complexes are activated by means of phosphorylation which is carried out by CDK activating kinases (CAK) and that, in turn, are negatively regulated by diverse CDK inhibitors (CDIs) (Toyoshima and Hunter, 1994; Sherr, 1996). The expression of cyclins, CDKs and CDIs, which is finely coordinated in non-pathological conditions, is often deregulated in tumours (Carracedo *et al*, 2008). The role played by p27 is that of a negative regulator of G0/G1 phase and a low expression of this protein is associated with disease progression (Hunter and Pines, 1994; Toyoshima and Hunter, 1994). Diverse studies report a down-regulation of p27 in OSCCs (Kudo *et al*,1998; Fujieda *et al*,1999; Mineta *et al*,1999; Singerland and Pagano, 2000; Shintani *et al*, 2002; Choi *et al*, 2003) accompanied by an overexpression of Skp2 (Gstaiger *et al*, 2001; Kudo *et al*, 2001; Shintani *et al*, 2003). Fewer studies have, however, investigated the cell migration upon which the metastatic capability of the tumour depends. A reduced p27 expression was found approximately in 87% of OSCCs and it is strongly associated with early tumour invasion, cell proliferation and metastatic potential (Kudo *et al*,1998; Mineta *et al*,1999; Shoelch *et al*,1999; Bloom and Pagano, 2003; Choi *et al*, 2003). The overexpression of p27 in healthy epithelia is well documented and known, whereas the oncoprotein Skp2 is poorly expressed. The reduction of p27 levels and the simultaneous increase of Skp2, which are thus inversely correlated, are indeed observable in epithelial dysplasia, OSCCs and early subepithelial tumour invasion (Jordan *et al*, 1998; Kudo *et al*, 2000, 2001; Gstaiger *et al*, 2001; Hershko *et al*, 2001; Latres *et al*, 2001; Masuda *et al*, 2002). Kudo hypothesised that the down-regulation of p27 may play a major role in the tumour invasion and metastasis processes mediated by the loss of cell-adhesion mechanisms (Kudo *et al*, 2000). However, the relation linking the down-regulation of p27 and metastasis remains unknown (St Croix *et al*, 1996).

In addition to this, a study conducted by Harada *et al* (2005a, 2005b) revealed that an Skp2 down-regulation actually induces apoptosis in OSSCs tumoural cells; furthermore, forcing the expression of Skp2 in quiescent fibroblasts induces DNA synthesis (Gstaiger *et al*, 2001). These results suggest that Skp2 possesses oncogenic properties.

The purpose of the present work was that of analysing the expression of Skp2 (20% cut-off) (Gstaiger *et al*, 2001; Harada *et al*, 2005a,b) and to correlate its tumoural levels with both the appearance of a regional nodal metastasis and prognosis in stage I and II OSCCs. Furthermore, the overall survival of the considered sample with respect to Skp2 expression was evaluated. Two different localizations of nodal spread were considered, so as to better evaluate the potency of Skp2 and to analyse its metastatic potential.

The overall survival of the sample of patients considered in this study was evaluated using Kaplan-Meier curves and data showed overall survival rates equal to 89.6%, 87% and 80.7% at 3, 5 and 10 years, respectively. The survival rates for early oral squamous cell carcinomas at 5 years which are reported in the literature range between 70 and 90% for tongue cancer and between 66 and 78% for carcinoma of the buccal mucosa (Diaz *et al*, 2003; Lin *et al*, 2006; Shaw *et al*, 2009).

The statistical analysis correlating survival curves and Skp2 expression that was carried out on our study sample showed a non-significant P value. However, an important element emerges from the present data: patients who presented Skp2 values greater than 20% deceased within 3 years from surgery. The previous studies demonstrated a strong correlation between Skp2 overexpression, metastatic capability of the tumour and a decrease in overall survival (Harada *et al*, 2005a,b; Carracedo *et al*, 2008).

Interesting results were obtained when analysing the relationship between Skp2 expression and both nodal and distant metastases. From our data one can gather that in those cases where Skp2 $\geq$ 20%, the cases of metastases (9 over 17) and no metastases (8 over 17) are similar in number. The situation changes when considering patients who presented Skp2 $<$ 20%, in which case a strict interrelation between the absence of metastases and such an Skp2 expression is noticeable. In fact, of the 54 patients with Skp2 $<$ 20%, 48 did not develop any nodal laterocervical metastasis, while seven of them did. Such a correlation resulted statistically significant with $P = 0.001$.

Apparently, just as literature reports for other tumours, high Skp2 levels play a predictive role also in OSCCs when considering the potential of the tumour to develop nodal metastases. This study is the first to report an important correlation between an Skp2 expression lower than 20% and the capability of the tumour not to develop nodal laterocervical metastases ($P = 0.001$).

Conclusions

The understanding and analysis of the expression of Skp2 have interested many studies in the last few years. However, further research is needed to better comprehend its action mechanisms and its predictive value for the development of metastases in oral squamous cell carcinoma. On the other hand, one should bear in mind the importance of deepening the knowledge on biotissue markers which could predict the metastatic behaviour of tumours. This could severely improve the therapeutic approach to OSCCs and possibly increase survival rates at 5 years of stage I-II carcinomas which is, at present, reported to range between 70 and 80%.

Author Contribution

All Authors equally contributed to this work

Competing financial interests statement

Nothing to disclose

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