Skp2, p27^{kip1} and EGFR assessment in head and neck squamous cell carcinoma: Prognostic implications

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Abstract. EGFR, p27^{kip1} and Skp2, have been implicated in human cancer development. We have studied these molecules in a search for molecular markers that may have prognostic value in head and neck squamous cell carcinomas (HNSCC). Tissue samples of 62 patients were collected and immunohistochemical analysis was carried out for p27^{kip1}, Skp2 and EGFR protein evaluation. Western blot analysis of p27^{kip1} was performed. The p27^{kip1} expression is frequently down-regulated in HNSCC (44.4%, 20/45 cases). The immunohistochemical analysis showed p27^{kip1} cytoplasmic retention in 7/38 tumors. Strong Skp2 signals were detected at the invasive edge of the tumor in cells lacking p27^{kip1} staining. We found a high EGFR staining in 49% (23/47) of the cases. The staining tended to be more frequent in lymph node-positive cases. The dysplastic tissue exhibited no Skp2 immunoreactivity, whereas 51.06% (24/47) of invasive tumors expressed high levels. Of note is that Skp2 overexpression was the only factor that significantly correlated with a shorter overall survival in multivariate analysis (p=0.048). Our results suggest that Skp2 is a useful prognostic marker for HNSCC management.

Introduction

Head and neck squamous cell carcinoma (HNSCC) remains a significant cause of morbidity and mortality, afflicting ~500,000 new cases worldwide each year (1). The clinical course of these tumors cannot be completely predicted by clinicopathological features. Therefore, new molecular markers that can be used for a more accurate clinical management of these tumors are needed (2).

Cell cycle progression is governed by cyclin-dependent kinases (cdks) that are regulated by phosphorylation, cyclin binding and cdk inhibitors. The coordinated expression of cyclins, cdks and cdk inhibitors is often deregulated in cancer. The cdk inhibitor p27^{kip1} regulates cellular progression from G_1 to S phase (3). The inactivation of p27^{kip1} has been implicated in the development of a broad range of human malignancies suggesting that it plays an important role in human cancer pathogenesis (4-11).

The regulation of p27^{kip1} expression occurs predominately at the post-translational level by a ubiquitin-proteasome-dependent degradation mechanism. Skp2 is a rate-limiting component of this machinery (12) and it plays a critical role in the regulation of p27^{kip1}, a key mediator of PTEN-induced G_1 arrest, through the PI 3-K/Akt/PTEN pathway (13). Skp2 overexpression has been observed in various types of human tumors (14). The p27^{kip1} and Skp2 levels are inversely correlated in colorectal cancer (15). These findings suggest that the enhanced p27^{kip1} degradation observed in many aggressive human tumors is attributable to increased levels of Skp2. However, not all aggressive cancers exhibit low p27^{kip1} expression levels. The inactivation of p27^{kip1} may also occur at cytoplasmic retention as a consequence of p27^{kip1} phosphorylation by Akt and its binding to 14-3-3 (16). This mechanism has been associated with a poor patient outcome in breast cancer (17). Additionally, the PI 3-K/PTEN/Akt pathway regulates p27^{kip1} protein stability at a transcriptional level. Akt is known to down-regulate p27^{kip1} transcription by the phosphorylation-dependent inhibition of the Forkhead family of transcription factors (18). Akt lies downstream of PI 3-K and EGFR, which act as oncogenes in numerous cancers, including HNSCC.

In a previous study, carried out in our laboratory, we determined the expression status of p110α (the catalytic subunit of PI 3-K), PTEN and pAkt in a series of HNSCC, and we identified this pathway as one of the most frequently altered in this type of tumor (19). These circumstances prompted us to examine the expression levels of p27^{kip1} and of molecules that may be important for its regulation in the context of HNSCC, such as Skp2 and EGFR. Their clinical significance

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and their potential utility as molecular prognostic markers for the clinical management of HNSCC patients were assessed.

Materials and methods

Patients. Surgical tissue specimens from 45 patients with HNSCC, who consecutively underwent resection of their tumors at the Hospital Universitario Central de Asturias (HUCA) between 2000 and 2003, were prospectively obtained for our study with institutional review board approval for guidelines on ethical procedures. Informed consent was obtained from each patient. No patient had received radio/chemotherapy prior to intervention or were thought to have distant metastasis at the time of diagnosis. Surgical samples were sharply excised, placed in sterile tubes and frozen immediately in liquid nitrogen. The cases were confirmed to be neoplastic by the pathologist. Additionally, paraffin-embedded tissues from 62 patients (including the 45 mentioned before) were obtained from Servicio de Anatomía Patologica at the same hospital. Information was obtained from clinical records.

The characteristics of the patients studied, the clinico-pathological features of their tumors [TNM staging system of the International Union Against Cancer (6th edition) (20)] and median follow-up periods (months) are shown in Table I. Two patients, who did not smoke or drink alcohol, were excluded from the analysis, to make the population more uniform. Thus, the patients in the study were regular tobacco and/or alcohol consumers. Twenty-eight patients were deceased at the end of follow-up.

Protein extraction and Western blot analysis. Samples were processed as previously described (19). Whole protein extracts (25 μg) from tumor and patient-matched normal epithelia were electrophoresed and immunoblotted with rabbit anti-human $p27^{kip}$ polyclonal antibody [Santa Cruz sc-528 (C-19)] (1:2000). Antirabbit immunoglobulin IgG secondary antibody was used (1:2000). For the protein load control, anti-ß-actin mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO) was used. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blot analysis system (ECL Plus, Amersham Pharmacia Biotech, Arlington Heights, IL).

Immunohistochemical analysis. Serial formalin-fixed paraffin-embedded sections (4 μm) were incubated overnight at 54-56˚C, deparaffinized in xylene and rehydrated through decreasing graded ethanol solutions. Endogenous peroxidase activity was suppressed by incubation for 10 min with the peroxidase block reagent (3% hydrogen peroxide) (Dako). After antigen retrieval (boiling in 10 mM citrate buffer, pH 6.0 for $p27^{kip}$, Skp2 or proteinase K treatment, for EGFR), the sections were processed on a Dako TechMate 500 immuno-stainer to obtain uniform staining. Non-specific staining was blocked with ChemMate™ wash buffer 1. Immunostainings were performed with the following specific primary antibodies: rabbit polyclonal antibody against human $p27^{kip}$ protein [Santa Cruz sc-528 (C-19) (1:750 dilution) (4˚C, overnight)], mouse monoclonal antibody against Skp2 (1G112E9, Zymed Laboratories Inc.) (1:100), mouse monoclonal antibody against an epitope on the extracellular domain of human EGFR (kit EGFR PharmDx™ K 1494, clon 2-18C9) (30 min). The sections were then incubated with the peroxidase-labelled polymer conjugated goat anti-rabbit/mouse secondary antibody for 30 min at room temperature. The stained proteins were visualized using the DAB solution provided in the kit, and lightly counterstained with haematoxylin.

Table I. Characteristics of the patient population and their tumors (N=62).

Feature	Frequency (%)
Gender	
Male	60 (96.77)
Female	2 (3.23)
Anatomic site	
Pharynx	40 (64.5)
Larynx	22 (35.5)
pT category	
T1	4 (6.45)
T2	13 (20.97)
T3	23 (37.1)
T4	22 (35.48)
pN category	
N0 (free margins)	24 (38.71)
N1-3	38 (61.29)
TNM stage[a]	
I	2 (3.23)
II	7 (11.29)
III	11 (17.74)
IV	42 (67.74)
TNM stage[a]	
I-III	20 (32.25)
IV	42 (67.75)
Histopathological grade	
Well	26 (41.94)
Moderate	27 (43.55)
Poor	9 (14.51)
Recurrence	
No	31 (50)
Yes	31 (50)
Mean age at resection (yrs)	59
Median age at resection (yrs)	61
Median length of follow-up (range)	
Total population	33 months (1-78)
Alive at last follow-up	67 months (48-78)
Deceased due to index tumor	18 months (4-60)
Deceased due to other causes	25 months (1-66)

[a]UICC, 6th edition.

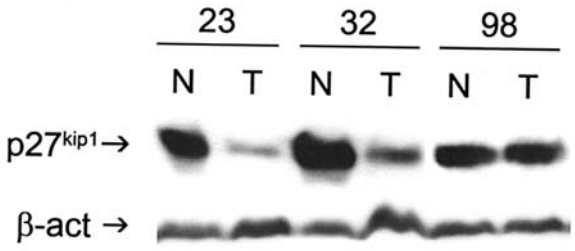

Figure 1. Western blot analysis of the p27^{kip1} protein expression in HNSCC. Whole cell lysates containing 25 μg of protein from tumors (T) and patient-matched normal epithelia (N) were subjected to Western blot analysis. The indicated proteins (bottom, ß-actin as the loading control) were detected using specific primary antibodies and were visualized using HRP-conjugated secondary antibodies as described in Materials and Methods. The case numbers are shown at the top.

To ascertain the specificity of the antibody immunoreactivity, negative controls were carried out by exclusion of the primary antibody. In this case, immunolabelling was completely abolished. Normal squamous epithelium, colon carcinoma tissue and infiltrating ductal breast carcinoma were also included as appropriate positive controls for p27^{kip}, EGFR and Skp2, respectively.

Each case was scored as a product of two parameters: the extent of immunostaining (on a 0-100 point scale, after counting cells in five random fields per sample with the help of the image tool 3.0 UTHSCSA application) and staining intensity (on a 1-4 point scale). Values ranged from 0 to 250 for p27^{kip1}, 0 to 249 for Skp2 and 100 to 300 for EGFR.

These continuous variables were categorized as low- and high-expressing tumors, according to the median values of the registered scores (30, 37 and 230 for p27^{kip1}, Skp2 and EGFR, respectively).

Statistical analysis. The clinicopathological features were dichotomized as follows: pT category, 1-3 vs. 4; pN category, 0 (free lymph nodes) vs. 1-3 (affected lymph nodes) and stage I-III vs. IV. The molecular data distributed among the different clinical groups of tumors were tested for significance by employing the χ^2 test, Student's t-test (two-tailed Mann-Whitney for non-parametrical variables) or ANOVA, where appropriate. A multivariate analysis (forward Wald method) was carried out to test the independent significance of molecular findings or clinicopathological parameters on the nodal status or local recurrence.

Survival curves were calculated using the Kaplan-Meier product limit estimate. Succumbing to causes other than the index tumor or its metastases were not considered treatment failures, and these patients were censored in any analysis involving the length of survival. Differences between the overall survival times were analyzed by the log-rank method. The multivariate Cox proportional hazard model was used to examine the relative impact of those variables demonstrated to be statistically significant in univariate analysis. Statistical analysis was carried out with the help of the software package SPSS 12.0 (SPSS, Inc., Chicago, IL). All tests were two-sided and $p<0.05$ values were considered to be statistically significant.

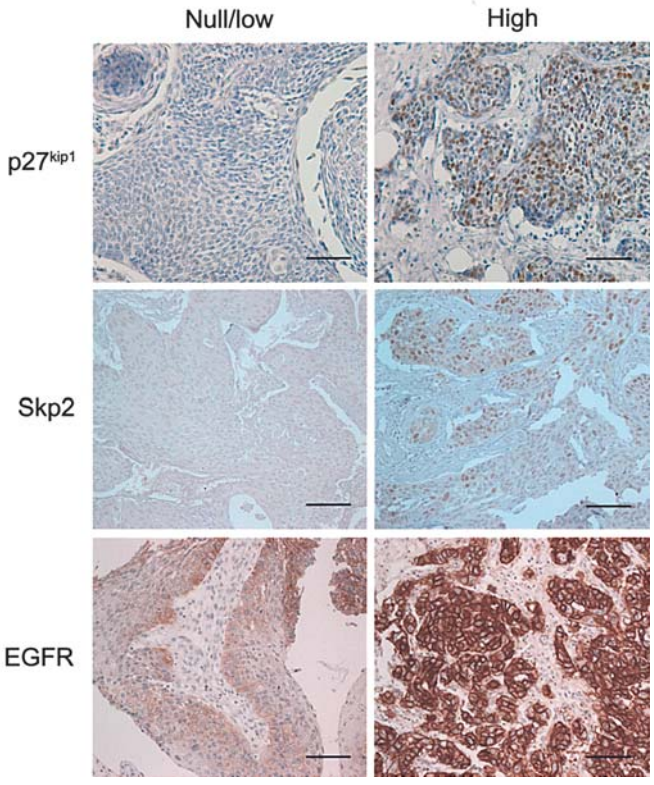

Figure 2. The immunohistochemical detection of p27^{kip1}, Skp2 and EGFR, showing null/low or high levels of protein expression. While p27^{kip1} and Skp2 display a nuclear pattern, EGFR expression is detected at the cell membrane; scale bar, 250 μm.

Results

p27^{kip1} protein expression is frequently reduced in HNSCC. Western blot analysis of p27^{kip} expression was performed in 45 paired samples of tumor and adjacent normal mucosa. A reduced p27^{kip1} expression was detected in 20 tumors (44.4%) (Fig. 1). This observation was confirmed by immunohistochemical analysis in 47 samples. The p27^{kip1} expression was scored as low in 40.43% (19/47) and high in 59.57% (28/47) (Fig. 2). The p27^{kip1} distribution in normal epithelium was detected as a strong positive signal in the most external layers that progressively diminished towards the actively dividing basal cells. Regarding p27^{kip1} subcellular localization, a nuclear staining pattern was observed in the p27^{kip1}-positive cases, and additional cytoplasmic p27^{kip1} was found in 7/38 tumors that showed any detectable p27^{kip1} signal. These cases showed a tendency toward higher p27^{kip1} levels (considered as a continuous variable) than those for which only nuclear localization was observed [U, 63 (p=0.075)].

Immunohistochemical comparison of the p27^{kip1}, Skp2 and EGFR protein expression in invasive HNSCC and dysplastic mucosa. In addition to the cytoplasmic retention, Skp2-dependent ubiquitination/degradation of p27^{kip1} constitutes another plausible mechanism to explain the reduced p27^{kip1} expression observed in our series of HNSCC or molecules such as EGFR (initiator of PI 3-K/Akt/PTEN pathway) being able to influence p27^{kip1} protein levels. Thus, we carried out

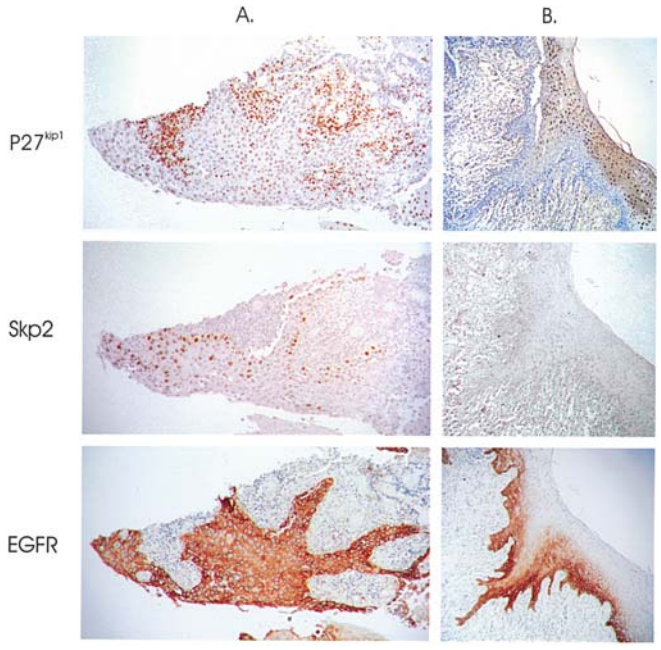

Figure 3. Immunohistochemical comparison of the $p27^{kip1}$, Skp2 and EGFR protein expression. (A) Consecutive tissue sections of a representative invasive HNSCC. $p27^{kip1}$ and Skp2 displayed an inverse pattern of cell distribution. A strong nuclear Skp2 signal is observed in cells located at the invasive edge of the tumor lacking $p27^{kip1}$-positive staining. The strong $p27^{kip1}$ immunoreactivity detected in infiltrated lymphocytes serves as a positive internal control. EGFR showed a membranous tumor-specific staining (no signal in lymphocytes). (B) Consecutive tissue sections of a representative premalignant dysplastic lesion. An absence of Skp2 immunoreactivity was observed in the dysplastic epithelium. $p27^{kip1}$ exhibited a positive signal in the most differentiated cell layers of the normal squamous epithelium that diminished toward the dysplastic areas, where EGFR immunoreactivity is higher (x45 magnification).

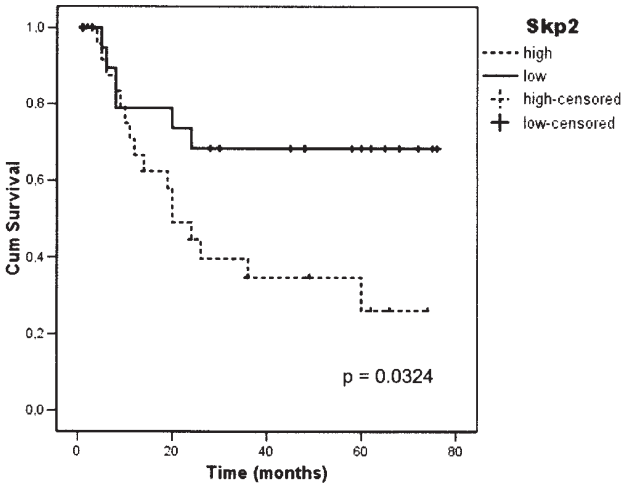

Figure 4. Kaplan-Meier disease-free survival curves of the patients with a low or high Skp2 immunoreactivity; p=0.0324, log-rank method.

an immunohistochemical analysis of Skp2 and EGFR. Tumors were classified as low or high Skp2-expressing in 23/47 (48.94%) and 24/47 (51.06%) cases, respectively. EGFR expression was found to be low in 24/47 (51%) and high in 23/47 (49%) of the cases (Fig. 2). To easily visualize and compare the sites and subcellular distribution, consecutive sections of a tumoral field were stained with $p27^{kip1}$, Skp2 and EGFR as shown in Fig. 3A. Strong EGFR membrane staining clearly delineates the tumor mass, which is negative in the infiltrated lymphocytes. This is in marked contrast to $p27^{kip1}$ immunostaining, which exhibits a nuclear pattern of low/moderate intensity in tumor cells and a strong positive staining in the lymphocytes. An inverse cellular distribution of $p27^{kip1}$ and Skp2 was observed in agreement with the postulated role of Skp2-mediating $p27^{kip1}$ degradation. The Skp2 signal localizes in the nucleus, with a stronger staining intensity at the invasive edge of the tumor in those cells lacking $p27^{kip1}$.

We also compared the changes in the expression pattern of $p27^{kip1}$, Skp2 and EGFR in the early stages of tumorigenesis. The consecutive sections of a representative premalignant dysplastic lesion stained with the same antibodies are shown in Fig. 3B. It is noteworthy that no Skp2 immunoreactivity was detected in any dysplastic squamous epithelium, whereas the staining patterns for the rest of the molecules resembled, in both level and distribution, those observed in invasive HNSCC.

Correlations of molecular findings with clinicopathological features and overall survival outcome. Cross-tabs between molecular findings and clinicopathological data are shown in Table II. There was a tendency toward higher EGFR levels in lymph node-positive cases [3.037 (p=0.081)].

Skp2 expression was higher in those cases with tumor recurrence [4.821 (p=0.013)]. While 36/40 (90%) pharyngeal tumors were stage IV, only 6/22 (27.3%) laryngeal tumors belonged to this group (p=0.000). The tumor site correlated with the positive lymph nodes: 30/40 (75%) pharyngeal tumors were lymph node-positive versus 8/22 (36.4%) with laryngeal localization (p=0.000).

The multivariate analysis (forward Wald method) demonstrated that only the anatomic site (pharynx vs. larynx) was associated with an increased risk of developing lymph node metastasis (0.001), excluding EGFR expression or the histopathological grade. However, none of the following factors: Skp2 expression, anatomic site, nodal status or tumor stage (which were statistically related to recurrence in univariate analysis) established an independent prognostic value for the incidence of local recurrence in multivariate analysis.

The five-year overall survival rate for the HNSCC cases with follow-up data was 38% (SE, 0.0952). The relationships between the protein expression levels of the studied markers and overall survival rates were examined. The cumulative survival rate of patients with Skp2 overexpression was significantly lower than that of the cases with low Skp2 expression [mean, 23 (±5) months vs. 45 (±5) months) [4.85 (0.0276)]]. Fig. 4 shows the Kaplan-Meier survival curves of the patients grouped by Skp2 immunoreactivity.

Similarly, patients with tumors arising in the pharynx presented a reduced cumulative survival rate when compared with laryngeal tumors [media of 31 (±5) vs. 54 (±5) months, 5.93 (0.0149)]. Other variables with an impact on overall survival rates were nodal status [5.46 (0.019)], and advanced tumor stage [7.17 (0.0074)]. These observations reveal that the tumor population under study is representative, since it follows the expected behaviour according to the well-established impact of these clinicopathological features on the overall survival of HNSCC patients.

Table II. Correlations of EGFR, $p27^{kip1}$ and Skp2 expression (IHC analysis) with clinicopathological findings.

	EGFR		$p27^{kip1}$		Skp2	
	Low	High	Low	High	Low	High
Anatomic site						
Pharynx	17	15	13	19	15	17
Larynx	7	8	6	9	8	7
p	0.680		0.968		0.680	
pT category						
T1	1	1	0	2	1	1
T2	5	5	4	6	7	3
T3	8	9	8	9	7	10
T4	10	8	7	11	8	10
p	0.967		0.640		0.507	
pN category						
N0	11	5	6	10	9	7
N1-3	13	18	13	18	14	17
p	0.081		0.769		0.471	
Histopathological grade						
Well	8	11	6	13	7	12
Moderate	12	9	8	13	11	10
Poor	4	3	5	2	5	2
p	0.599		0.177		0.269	
TNM stage						
I-III	7	6	7	6	8	5
IV	17	17	12	22	15	19
p	0.813		0.246		0.285	
Recurrence						
No	12	10	8	14	15	7
Yes	12	13	11	14	8	17
p	0.654		0.590		0.013	

p, χ^2 test.

A multivariate model using Cox regression analysis (forward stepwise method) was used to evaluate the statistical strength of independent associations between covariates and survival. The only parameter with independent prognostic value was Skp2 expression of the primary tumor (p=0.048; 95% CI, 1.011-6.911) when it was tested along with the variables of anatomic site (p=0.139), nodal status (p=0.155) and tumor stage (p=0.207).

Discussion

The abundance of $p27^{kip1}$ is controlled in a cell-type and signal-specific manner by the integration of mitogenic and antimitogenic signaling pathways that affect its translation, stability and localization. The inactivation of $p27^{kip1}$ can be achieved by a number of different oncogenic stimuli, including the loss of PTEN, up-regulation of EGFR, Skp2 or Akt activity. The fact that most of the control of $p27^{kip1}$ abundance is post-transcriptional suggests that a proteomic survey would be an adequate approach to study $p27^{kip1}$ expression and its potential prognostic value (21). To accomplish this, we performed a $p27^{kip1}$ protein analysis using immunohistochemistry and Western blot analysis. The two techniques revealed a similar proportion of tumors with reduced $p27^{kip1}$ levels (40.43 and 44.4%, respectively).

In breast cancer, where reduced $p27^{kip1}$ levels constitute an independent poor prognostic indicator (4), it has been suggested that its accelerated proteolysis reflects an increased PI 3-K activity (16). We did not find any association between the $p27^{kip1}$ down-regulation and alterations of the PI 3-K/Akt pathway [detected in a previous study as pAkt, or p110α accumulation or PTEN down-regulation (19)], a similar result to that reported in thyroid cancer (22). Additionally, this is the first report showing $p27^{kip1}$ cytoplasmic retention in HNSCC (7/38 cases). Although it has been described that the cytoplasmic localization of $p27^{kip1}$ predicts a poor prognosis

in late-stage ovarian carcinoma (23), we did not observe any impact on prognosis in our HNSCC series. Moreover, no relationship was established between $p27^{kip1}$ mislocalization and alterations of the PI 3-K/Akt pathway. This result is in agreement with other reports in glioblastoma cells showing that $p27^{kip1}$ cytoplasmic localization does not change in response to PI 3-K activity (24).

A high Skp2 expression was observed in 51.06% of the cases and an inverse expression pattern to $p27^{kip1}$ immunostaining was observed in certain tumor areas (Fig. 3A). No correlation was found between Skp2 expression and any PI 3-K/Akt alteration. Significantly, Skp2 immunoreactivity was not detected in premalignant lesions that are often found in the vicinity of tumor tissues (Fig. 3B). This suggests that Skp2 overexpression accompanies tumorigenesis. Moreover, Skp2 is associated with tumor recurrence (p=0.013) and with a shorter cumulative survival rate (p=0.032). Notably, Skp2 was the only independent prognostic factor on a multivariate Cox model basis (p=0.048). These results are in agreement with those obtained by Kudo *et al* (25), suggesting that Spk2 is a novel marker of poor prognosis in HNSCC management.

EGFR is the target of antitumoral drugs, such as erlotinib. Its therapeutic activity results from EGFR inhibition followed by $p27^{kip1}$ up-regulation, as has recently been demonstrated in NSCLC cell lines (26). In HNSCC, a high expression of EGFR has been described, although its association with disease outcome remains controversial (27,28). In our HNSCC series we detected high EGFR immunostaining in 49% of the cases. EGFR immunostaining tended to be more frequent in lymph node-positive cases (p=0.081). It has been reported that EGFR inhibition blocks the proliferation and survival of cancer cells and this is associated with an increased $p27^{kip1}$ expression, suggesting a mechanistic connection between these two events (29). In agreement with this idea, Fig. 3A shows an inverse pattern of EGFR and $p27^{kip1}$ staining, along with a partially coincidental pattern of the Skp2 protein.

As expected, tumors arising at the pharynx presented lymph node involvement (p=0.000), a more advanced global disease stage and a poorer prognosis (p=0.000) more frequently than laryngeal tumors. Taken together, these data indicate that the tumor population under study is representative and shows the expected behavior.

This report points to a Skp2 protein level immunohistochemical evaluation as a potential marker of poor prognosis for head and neck squamous cell carcinoma. Further studies validating these results on an independent HNSCC population are warranted.

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