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# CDC28 protein kinase regulatory subunit 1B (CKS1B) expression and genetic status analysis in oral squamous cell carcinoma

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**Summary.** CKS1B is a member of the highly conserved cyclin kinase subunit 1 (CKS1) protein family which interacts with cyclin-dependent kinases and plays a critical role in cell cycle progression. In oral squamous cell carcinoma (OSCC), as in other malignancies, CKS1B overexpression has been correlated with reduced survival. To our knowledge, no studies evaluating the genetic status of *CKS1B* gene in OSCC have been reported. Herein, genetic and protein status of CKS1B were analyzed by immunohistochemical (IHC) and fluorescence *in situ* hybridization (FISH) techniques in a series of primary OSCC (n=51) and lymph node OSCC metastases samples (n=14). The observed results were compared with those obtained in either inflammatory (oral lichen planus [OLP]) (n=13) and premalignant oral mucosal lesions (oral leukoplakia) (n=16). A significant CKS1B overexpression was observed in OSCC and lymph node metastases samples than in OLP and oral leukoplakia (mean 70% vs 35%, p<0.001). CKS1B overexpression correlated with p27 loss of expression (p=0.0013) and SKP2 overexpression (p<0.00). FISH study disclosed statistical differences in both gene amplifications and gains between samples corresponding to OSCC and metastases from those of OLP and leukoplakia (p<0.001). Amplifications were present in 53% of OSCC samples and 33% of lymph node metastases vs 14% of oral leukoplakia and 0% of OLP biopsy specimens (p=0.002). Polysomies of chromosome 1 were seen in 46% of OSCC, 33% of ganglionar metastases, 14% of oral leukoplakia and 10% of OLP (p=0.036). Correlation of CKS1B over-expression and gains (both polysomies and amplifications) determined by FISH was statistically significant (p<0.001).

Our results indicate that a high CKS1B expression is a common finding in primary OSCC which correlates with p27 low expression and SKP2 overexpression. This phenomenon may be due either to numerical (chromosome 1 polysomy) or structural (amplifications) *CKS1B* genetic abnormalities. This phenotypical and cytogenetic profile is not observed in premalignant or inflammatory oral mucosal lesions.

**Key words:** Carcinogenesis, p27, *CKS1B*, FISH, Squamous cell carcinoma, Oral leukoplakia, Lichen planus

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## Introduction

Oncogenesis in the oral cavity is widely believed to result from cumulative genetic alterations (Califano et al., 1996) that cause a step-wise mucosal transformation from normal to dysplastic and to invasive carcinoma, usually as a result of exposure to environmental agents (such as alcohol, tobacco and human papillomavirus) (Forastiere et al., 2001).

Different technical approaches have been used to analyze chromosomal abnormalities in oral squamous cell carcinoma (OSCC), such as fluorescent *in situ*

hybridization (FISH), comparative genomic hybridization (CGH) and CGH array. The observed karyotypes are complex and may contain different numerical and structural abnormalities, including balanced and unbalanced translocations, deletions, dicentric chromosomes and gene amplifications (Reshmi et al., 2004). Structural rearrangements affecting centromeric and pericentromeric regions, leading to the formation of isochromosomes, are characteristic features. Alteration of Y chromosome, activation of several proto-oncogenes, such as *CCND1, MYC, RAS, EFGR* and inactivation of suppressor genes such as *P16* and *TP53*, among others, have been described. The most frequently observed chromosomal alterations are losses of 3p, 8p, 9p, 11q, 13p, 14p and 15p; and gains affecting 1q, 3q, 8q and 15q chromosomes (Tsantoulis et al., 2007).

CKS1B is a member of the highly conserved cyclin kinase subunit 1 (CKS1) protein family which interacts with cyclin-dependent kinases (Cdks) and plays a critical role in cell cycle progression (Hadwiger et al., 1989). It is also an essential cofactor for efficient Skp2-dependent ubiquitinylation of $p27^{kip1}$ (Ganoth et al., 2001; Spruck et al., 2001). CKS1B overexpression is correlated with low p27 expression and adverse survival in several malignancies, including multiple myeloma, gastric, colorectal and head and neck squamous cell carcinomas (Matsuda et al., 2003; Kitajima et al., 2004; Shapira et al., 2004; Chang et al., 2006).

The aim of the present study was to analyze the genetic and protein status of *CKS1B* by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) in a large series of OSCC patients.

## Materials and methods

### *Patients*

Fifty-one patients presenting OSCC (six corresponding to T1 neoplasms, 23 to T2, five to T3 and 13 to T4), 14 of them presenting regional lymph node metastases, along with 13 patients with oral lichen planus (OLP) and 16 oral leukoplakias were included in the study. In all cases of oral lichen planus, along with characteristic clinical features, a mucous biopsy specimen showing diagnostic features was previously obtained. The diagnosis of oral leukoplakia was established in clinical white oral patches showing histologically epithelial dysplasia including architectural disturbance and cytological atypia.

Following a systematized protocol the clinical features of all patients included in the study were reviewed (Table 1). The study was approved by the local committee and written informed consent was obtained from all patients according to the Declaration of Helsinki.

The evaluated samples, collected between 1989 and 2007, were retrieved from the Pathology Departments of Hospital del Mar and Hospital de la Santa Creu i Sant Pau from Barcelona and Consorci Sanitari Parc Taulí, from Sabadell, Spain. Samples included: oral mucosal biopsies corresponding to OSCC (n=51), lymph node biopsies showing metastatic spread of OSCC (n=14), OLP biopsy specimens (n=13) and oral mucosal biopsies diagnosed as oral leukoplakia (n=16), according to the previously defined criteria. In addition, 14 samples corresponding to normal oral mucosa were also evaluated as a control group. Clinical and clinicopathological characteristics are summarized in Table 1 and Table 2, respectively.

**Table 1.** Clinical characteristics of the patients whose samples were included in the study.

| | OSCC | Oral Leukoplakia | OLP |
|---|---|---|---|
| Mean Age | 65 (43-92) | 65 (43-90) | 70 (45-94) |
| M/W | 41/10 | 9/7 | 7/6 |
| Tobacco Use | 31 (66%) | 16 (100%) | 6 (43%) |
| Alcohol abuse | 24 (51%) | 5 (31%) | 1 (7%) |
| Preexisting lichen planus | 3 (6%) | 1 (6%) | - |
| erosive | 2 | 1 | 3 |
| reticulate | 1 | 0 | 10 |

OSCC: oral squamous cell carcinoma; OLP: oral lichen planus; M/W: male/women.

**Table 2.** Clinicopathological characteristics of the patients with OSCC whose samples were included in the study.

| Clinicopathologic parameter | Nº samples (n = 51) / (%) |
|---|---|
| Tumor stage | |
| T1 | 6 (12%) |
| T2 | 23 (49%) |
| T3 | 5 (11%) |
| T4 | 13 (28%) |
| Localization | |
| Tongue | 21 (41%) |
| Floor of the mouth | 11 (21) |
| Alveolar ridge | 3 (6%) |
| Retromolar trigone | 5 (10%) |
| Buccal mucosa | 5 (10%) |
| Uvula | 4 (8%) |
| Hard palate | 2 (4%) |
| Lymph node stage | |
| N0 | 29 (62%) |
| > or equal N1 | 18 (38%) |
| Grade | |
| Well differentiated | 12 (25%) |
| Moderate | 28 (60%) |
| Poorly differentiated | 7 (15%) |
| Recurrence | |
| Yes | 16 (34%) |
| No | 31 (66%) |
| Died of the disease | |
| Yes | 13 (27%) |
| No | 34 (73%) |

### *Tissue microarrays (TMA)*

Two tissue microarrays (TMA) were constructed as outlined by Kononen et al. (1998) (Beecher Instruments, Silver Springs, MD, USA). A representative area of each tumour was selected in the donor paraffin blocks and a duplicated core of 1 mm of diameter was included in the final TMA. Serial 3-4 $\mu$m sections were cut and stored until FISH and IHC analysis were performed.

### *Immunohistochemistry (IHC)*

The Dakoautostainer immunostainer (Dako, Glostrup, Denmark) was used for immunohistochemical staining for CKS1B antibody (Zymed Inc, 1:50 dilution), SKP2 (Santa Cruz, 1:25 dilution) and p27 (BD Biosciences, Philadelphia PA, USA, dilution 1:100). The detection system employed was EnVision Plus (DakoCytomation). CKS1B immunohistochemical expression was scored according to the percentage of tumor cells exhibiting nuclear staining. In order to establish a level of significance of CKS1B expression in biopsy specimens, an empirical cut-off level corresponding to the median of CKS1B expression observed in normal mucosal samples plus one standard deviation was established. Positive or high CKS1B expression was only considered when more than 60% of the nuclei of tumor cells showed positive staining (overexpression).

p27 and SKP2 scores were measured as a percentage of tumor nuclei showing positive stain and were classified as high staining (>30% of nuclei), moderate (5 to 30%) or low (when staining was in 5% of nuclei).

### *Fluorescence in situ hybridization (FISH)*

Status of *CKS1B* gene by FISH was performed using a RP11-139I14 BAC clone mapping the *CKS1B* locus on chromosome 1q21.2 from Children's Hospital Oakland Research Institute library (Oakland, CA, USA). DNA isolation was performed according to Qiagen plasmid MIDI kit protocol. DNA was labelled using nick translation kit (Abbott Molecular, Abbott Park, IL, USA) with the Spectrum Green d-UTP (Abbott Molecular). The cytogenetic localization of all BACs was verified by hybridization to normal metaphase chromosomes (G-banding with inverted DAPI). Moreover, a centromeric probe for chromosome 1 (CEP 1 (D1Z5) SpectrumOrange™ Probe, Abbott Molecular) was used as control of polysomy or amplification. A total of 100 non-overlapping nuclei per sample were analyzed. Fourteen normal mucosa samples from healthy donors were used as negative controls. These samples were hybridized with the same probe to establish the cut-off values calculated as the mean of false-positive findings plus three times the standard deviation. One hundred nuclei per case were scored by two different observers taking into account the following abnormal FISH patterns: monosomy (one 1q21 and one CEP1 signal), amplification (1q21 signal/centromeric signal ratio greater than 2 per cell) and polysomy (three or more signals of 1q21 and CEP1). The calculated values for these patterns in control tissues were 45%, 4%, and 2%, respectively.

### *Statistical Analysis*

For the comparison of different groups, statistical correlations were carried out using Pearson's chi-square or Fisher's exact test depending on the nature of data. A P-value of $<0.05$ was considered as statistically significant. All statistical analyses were performed with SPSS 15.0 (SPSS Inc, Chicago, IL).

## Results

CKS1B overexpression was detected in 39 out of 51 (77%) biopsy specimens of OSCC (Fig. 1) and in 6 of 14 (46%) cases of lymph node metastases. Conversely, no cases included in groups of OLP and leukoplakia showed high CKS1B expression. A significantly higher CKS1B expression was observed in OSCC and lymph node metastasis ($p<0.001$) when compared with benign oral lesions (Table 3).

P27 expression was found to stain the nuclei from the upper layers of the epithelia, being negative in the basal layer, in all the cases of normal mucosae, OLP and oral leukoplakia studied. All these cases show moderate to high staining. However, in OSCC samples and lymph node metastases its expression was considered to be low in of 30 out of 49 samples (61.2%).

SKP2 expression was confined to the basal layers in

**Table 3.** CKS1B expression in the different groups studied.

| CKS1B | OSCC n=51 | Ganglionar M1 n=14 | Oral Leukoplakia n=16 | OLP n=13 | P value |
|---|---|---|---|---|---|
| % high expression by IHC<br>mean, range, sd | 39 (77%)<br>70%; 0-100%; 21% | 6 (46%)<br>50%; 0-90%; 29% | 0 (0%)<br>30%; 0-50%; 45% | 0 (0%)<br>35%; 0-60%; 15% | <0.001 |
| % gene amplification by FISH | 21 (53%) | 5 (33%) | 1 (14%) | 0 (0%) | 0.002 |
| % polysomies by FISH | 18 (46%) | 5 (33%) | 1 (14%) | 1 (10%) | 0.036 |

sd: standard deviation; IHC: immunohistochemistry; FISH: fluorescence in situ hybridization; OLP: oral lichen planus; OSCC: oral squamous cell carcinoma.

normal mucoase and OLP samples. In OLK, 7 out of 9 samples (77.7%) showed only basal stain. In two cases more than 50% of tumoral cells exhibited staining. In OSCC and lymph node metastases samples, 48 out of 51 (94%) samples exhibited positive staining, whereas only three samples showed low staining. In Fig. 2, immunohistochemical satining for CKS1B, P27 and SKP2 is shown.

Correlation between CKS1B overexpression and p27 low expression and SKP2 overexpression was studied, and was statistically significant (p=0.0013 and p<0.000 respectively) (Table 4). CKS1B overexpression was observed in 31.2% of the cases that show low expression of p27 vs 15.6% of the cases of moderate to high p27 expression.

FISH study disclosed *CKS1B* gene amplifications (Fig. 3) in 53% of OSCC samples and in 33% of lymph node metastases. In contrast, this alteration could only be observed in 14% of oral leukoplakia samples and it was absent in cases of OLP.

Polysomies (including trisomies, tetrasomies, or more) were seen in 46% of OSCC, 33% of lymph node metastases, 14% of oral leukoplakia cases and 10% of OLP. Statistical differences were seen both in the analysis of the presence of gene amplifications and gains, in the groups of OSCC and lymph node metastases vs oral leukoplakia, OLP and healthy mucosae (p<0.001).

Correlation of CKS1B overexpression and gains (both polysomies and amplifications) determined by FISH was statistically significant (p<0.001).

CKS1B overexpression was not related to OSCC histopathological grade, tumor stage (T1 and T2 vs T3 and T4), presence/absence of lymph node metastases,

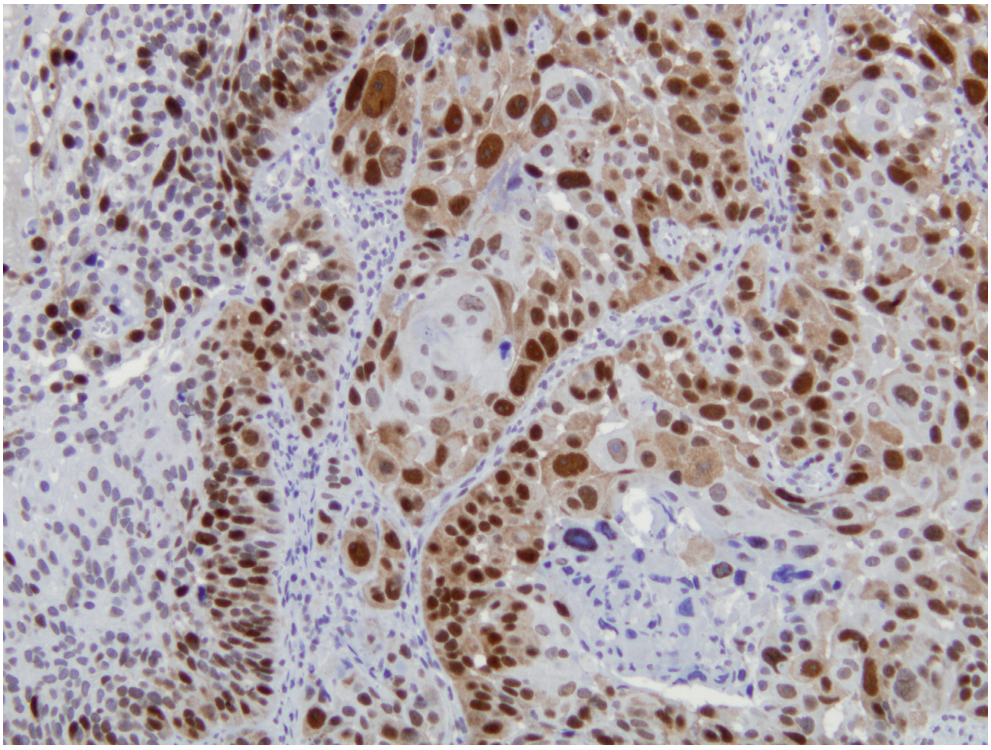

**Fig. 1.** OSCC tumoral cells with a high expression (>60% of tumour cells) of CKS1B. x100

**Table 4.** Correlation between CKS1B and p27 expression.

| | | CKS1B expression | |
|---|---|---|---|
| | | Low | High |
| P27 expression | Low | 12.3% | 31.2% |
| | Moderate and High | 41% | 15.5% |
| *P*=0.0013 | | | |

| | | CKS1B expression | |
|---|---|---|---|
| | | Low | High |
| SKP2 expression | Low | 75% | 37.5% |
| | Moderate and High | 11.4% | 88.6% |
| *P*<0.000 | | | |

**Table 5.** Correlation between overexpression and gene amplification/gains and clinicopathological course of OSCC.

| CKS1B | Clinicopathological course | | | | |
|---|---|---|---|---|---|
| | Grade I+ II vs III | T1+T2 vs T3+T4 | N0 vs N1-3 | Relapse No vs Yes | Exitus No vs Yes |
| % high expression by IHC | 29(85%) vs 6100%) | 19(82%) vs 16(94%) | 23(88%) vs 12(85%) | 24(88%) vs 11(84%) | 27(90%) vs 8(80%) |
| *P* | 1.000 | 0.373 | 1.000 | 1.000 | 0.584 |
| % gene amplification by FISH | 20(69%) vs 4(66%) | 15(68%) vs 9(69%) | 16(73%) vs 8(66%) | 16(73%) vs 8(61%) | 18(72%) vs 6(66%) |
| *P* | 1.000 | 1.000 | 0.714 | 0.708 | 1.000 |
| % polysomies by FISH | 16(55%) vs 4(66%) | 12(54%) vs 8(61%) | 14(61%) vs 6(50%) | 13(62%) vs 6(42%) | 16(61%) vs 4(44%) |
| *P* | 0.680 | 0.686 | 0.537 | 0.268 | 0.451 |

IHC: immunohistochemistry; FISH: Fluorescence in situ hybridization.

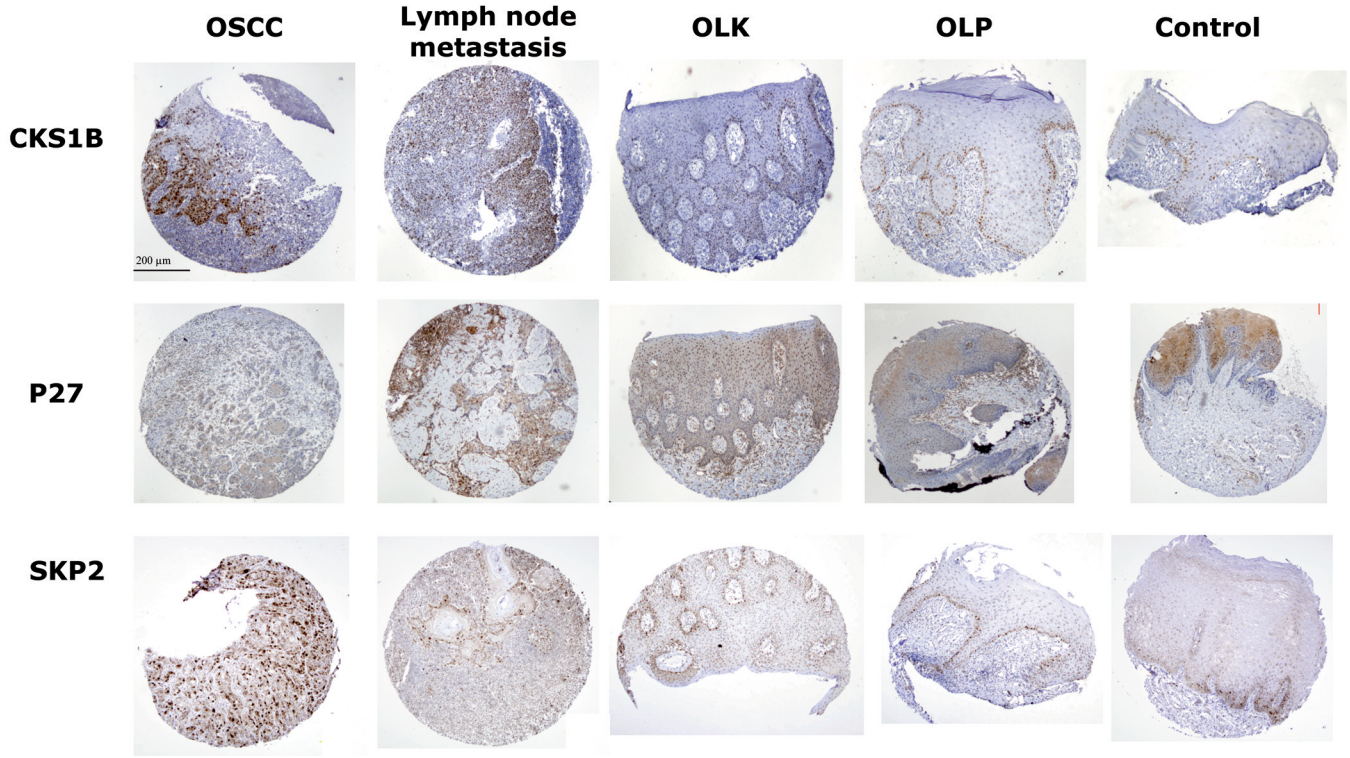


**Fig. 2.** CKS1B, P27 and SKP2 immunistochemical staining in OSCC, lymph node metastases, OLK, OLP and healthy mucosae of the TMA constructed for the present study. Note the high expression of CKS1B and SKP2 in malignant samples when compared to benign samples. Conversely, P27 stain is higher in healthy mucosae than the OSCC sample.

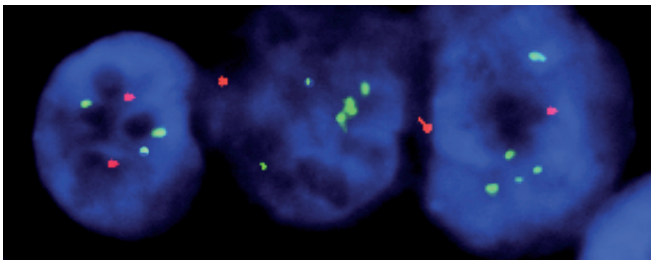

**Fig. 3.** Three OSCC cells from one sample evaluated by FISH. Most nuclei show multiple green CKS1B probes and fewer orange centromeric probes, showing amplification of 1q21.2.

relapse of the local illness and death due to the illness. Gains or amplifications were not statistically related to any of these clinicopathological markers (Table 5).

## Discussion

A large variety of chromosomal aberrations (Patel et al., 2001) have been reported in oral cancer. The impact of these aberrations varies significantly and their cellular and clinical significance is frequently uncertain. The number of chromosomal aberrations seems to increase

steadily during cancer progression. Oral leukoplakia has fewer aberrations than OSCC and lower tumor stage is associated with fewer abnormalities than higher tumor stage.

Some of these aberrations seem to be more prevalent in pre-malignant lesions or early stage oral cancer. In early oral neoplastic lesions, loss of heterozygocity or homozygous deletions in 3p, 9p, 13q and 17p have been reported. These aberrations may be linked to genes like *TP53* in 17p13, *RB1* in 13q14 or the *CDKN2A* gene in 9p21(encoding for p16 and p14 cyclin-dependent-kinase inhibitors) (Tsantoulis et al., 2007). Several CGH studies have reported some aberrations that are usually associated with advanced tumor stage or poor differentiation including allelic losses in 5q, 22q, 4q, 11q, 18q and 21q. Gains on 3q26 and 11q13 and deletions on 8p23 and 22q could also be valuable markers of an aggressive disease (Patmore et al., 2005).

*CKS1B* gene maps to chromosome region 1q21.2, which is frequently amplified in a variety of solid tumors (Chu et al., 2001), including head and neck squamous cell carcinoma (Bockmuhl et al., 2002). *CKS1B* seems to act as an adaptor protein which promotes p27 ubiquitination similarly to SKP2, thus inducing p27 degradation. Both the overexpression of CKS1B and SKP2 promote the proteolysis of p27, which is considered a putative tumor suppressor, so a loss in p27 may lead to the uncontrolled proliferation of malignant cells (Lloyd et al., 1999). *CKS1B* overexpression correlates with p27 reduced expression (Matsuda et al., 2003). Several studies have shown a loss in p27 protein in aggressive cancers such as breast cancer, prostate cancer, hepatocellular carcinoma, gastric, non-small cell lung and colorectal carcinomas (Inui et al., 2003; Matsuda et al., 2003; Shapira et al., 2004; Porter et al., 2006). P27 loss has also been related with drug resistance disease (Abukhdeir and Park, 2008), which could be of therapeutic interest.

Concerning CKS1B protein expression, we found overexpression in 77% of OSCC samples and 46% lymph node metastases, being absent in oral leukoplakias and OLP. However, no correlation with the clinicopathological stage could be found. In addition, a significant relationship between CKS1B overexpression and lower p27 expression and SKP2 overexpression was detected. Kitajima et al. (2004) found high expression of CKS1B in 62% of 63 cases of OSCC, but in 0% of normal oral mucosa. High expression of CKS1B was associated with low expression of p27, but no statistical correlation could be demonstrated. In contrast to our study, the cut off level of CKS1B expression was established at 30%. Fourteen normal oral tissue samples were used as negative controls. Our control samples did show higher expression of CKS1B, which ranged from 0% to 60% (median 35, mean 27, standard deviation 24). Thus, our cut off was established in a 60% of positive cells per sample.

To our knowledge, the *CKS1B* genetic status has not been described in OSCC before. Our study demonstrates that amplifications of this gene are a common event in OSCC when compared to healthy mucosa, OLP and oral leukoplakia.

Interestingly, as they were absent in OLP and present in only 10% of oral leukoplakia samples, CKS1B amplification could be considered as a marker of malignancy.

Correlation between CKS1B expression and genomic gains detected by FISH has been shown in the present study, suggesting that CKS1B overexpression may be attributable to gene amplification and/or polysomies of chromosome 1.

This phenomenon is similar to that reported in HER2/neu gene in breast cancers or EGFR gene in lung tumors, where overexpression is attributable to gene aberration. Statistical differences in CKS1B overexpression between OSCC and lymph node metastases were detected. Conversely, these differences were not confirmed by FISH analysis.

In conclusion, our results show that an increased CKS1B expression and a low p27 expression are common phenomena in OSCC, being absent in oral leukoplasias and benign oral inflammatory disorders. CKS1B overexpression is probably the consequence of either gene amplification or chromosome 1 polysomy. Further studies are warranted in order to confirm the practical diagnostic value of *CKS1B* assessment (gene and protein status) in OSCC.

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