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# Adhesion Molecule CD44 as a Prognostic Factor in Tongue Cancer

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**Abstract.** *Background: Loss of expression of CD44 has been shown to be a factor of poor prognosis in some types of tumors. The purpose of this study was to analyze this in relation to the survival of patients with tongue cancer. Materials and Methods: The expression of adhesion molecule CD44 was studied in 56 patients with tongue cancer. Data were gathered on clinical (T, N, M and stage) and pathological (T, N, stage, extracapsular spread, differentiation, tumor thickness, surgical margin and CD44 expression) parameters. Immunohistochemistry studies were carried out using DF1485 anti-CD44 monoclonal antibody. Results: In five tumors (8.9%) <25%, in 11 (19.6%) 25%-49%, in 15 (26.8%) 50%-74% and in 25 (44.6%) ≥75% of the neoplastic cells expressed CD44. A Cox proportional risks multivariate analysis identified CD44 expression as the parameter most associated with survival ($p<0.001$). Conclusion: The reduced expression of CD44 behaves as a marker of poor tongue tumor prognosis.*

Oral squamous cell carcinoma is a common cancer that often courses with an unfavorable prognosis, despite its localization in an easily-explored area. Among different intraoral localizations, the tongue is associated with an especially poor prognosis (1). Study of the prognostic factors that influence the survival of patients with tongue cancer is, therefore, of great interest. The prognostic value of the TNM system has been widely reported to be inadequate in patients with oral squamous cell carcinoma (2-4) and efforts have been made to identify clinico-pathological and molecular parameters (5-7) that enable the prognosis of these patients to be objectively predicted.

Little is known about the molecular mechanisms underlying the infiltration and destruction of adjacent tissues and the metastatic expansion of tumor cells (8). One precondition may be a change in the expression of intercellular adhesion molecules expressed on the tumor cell surface (9-11). The CD44 molecule has been shown to be a major factor in cell-cell interactions and cell adhesion. The standard form of this molecule is CD44s and there are variant isoforms (CD44v) derived from post-transcriptional splicing of the mRNA of the CD44 gene (12,13). It seems reasonable to hypothesize that the loss of CD44 expression releases neoplastic cells from their adhesion to neighboring cells, favoring their invasiveness. Therefore, loss of expression of this molecule should behave as a factor for poor tumor prognosis. Some studies of oral cancer support this hypothesis (8,14). The present study analyzed the influence of the tumor expression of CD44s on the survival of patients with tongue cancer.

## Patients and Methods

A survival study was conducted of 81 patients with tongue cancer treated at the University Hospital of Granada, Spain, from 1990 to 1996, and followed up until 1998. The same series was previously used for an investigation into the influence of tumor thickness on survival (5). Immunohistochemical methods could be used to assess CD44 expression in 56 of these patients, who constituted the study sample for the present analysis. The mean age of these 56 patients was 57.9 years (range 37-87 years) and 45 were male.

The clinical data of the patients were obtained from hospital medical records and included the T parameter value, the increase in cervical lymph node involvement determined by clinical methods (N) and the presence of distant metastasis according to IUAC and AJCC criteria (15).

The measurement of the pathologic T parameter was obtained from the pathology report in the medical records of the patients.

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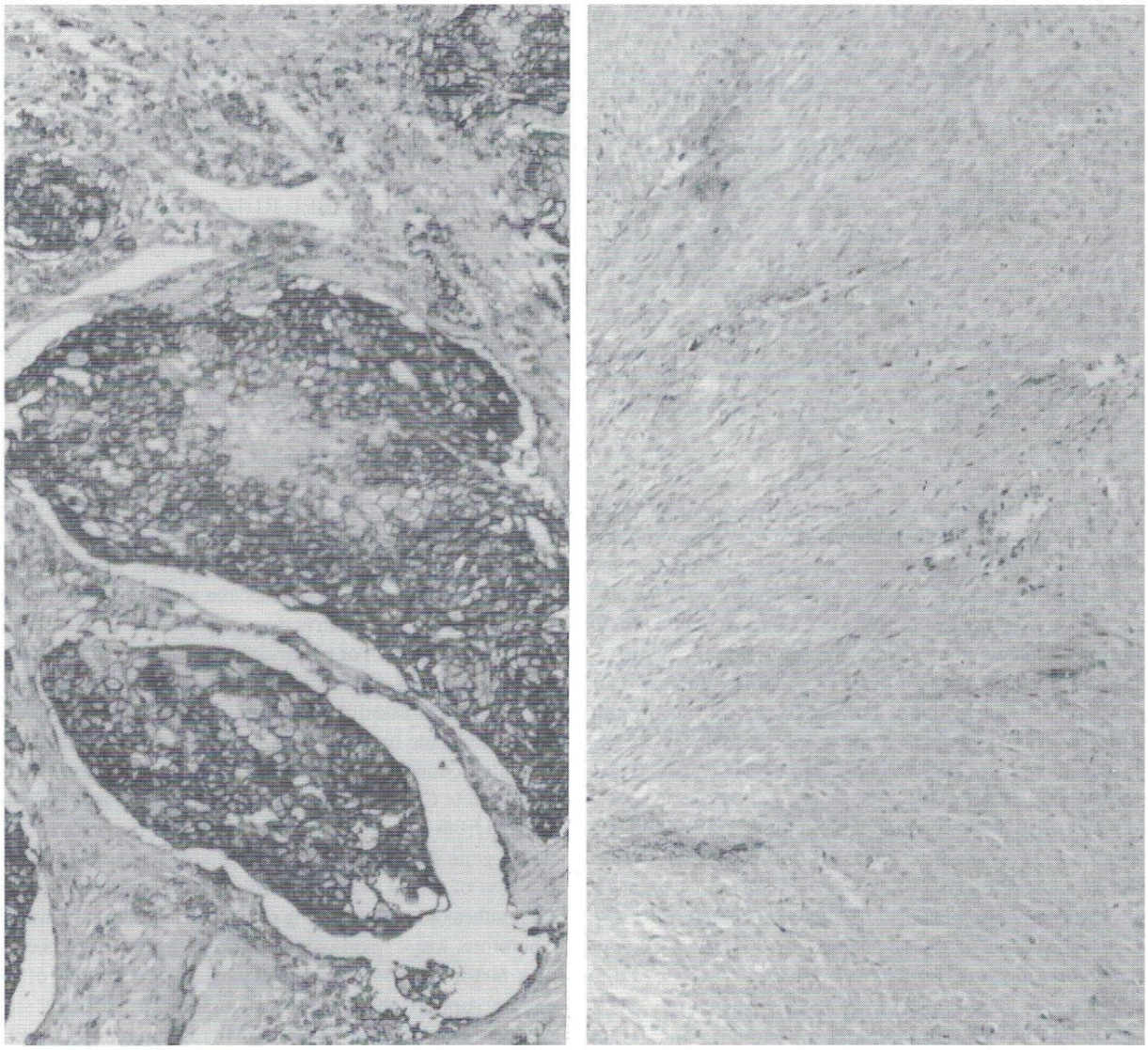

Figure 1. *Positive (A) and negative (B) CD44-expression in tongue cancer. Hematoxylin-eosin staining of formalin-fixed and paraffin-embedded operative tissue sections (10x).*

The histopathological data and the measurement of the tumor thickness were obtained by hematoxylin-eosin staining of formalin-fixed and paraffin-embedded operative tissue sections. The tumor involvement of cervical lymph nodes (pathologic N) was assessed according to IUAC and AJCC criteria (16). The degree of differentiation and the status of the surgical margin were also evaluated. The tumor thickness was measured using a method reported elsewhere (5). The histopathological studies were all carried out by a single pathologist (IRA).

For the immunohistochemical staining, 4 μm sections were cut from the paraffin blocks. After blockage of endogenous peroxidase with $H_2O_2$ in methanol for 30 min, sections were immersed in citrate buffer (pH 6.0) in a microwave-resistant container. The anti-CD44 antibody used was Clone DF1485 from Dako (Dako Corporation, Carpinteria, CA, USA). Sections were incubated overnight. Immunoperoxidase detection was employed using the ABC method (Dako) and diaminobenzidine substrate. Counter-staining was performed with hematoxylin. Antigen retrieval methods were used in this study. Staining of infiltrating lymphocytes was considered as positive internal control. A carcinoma section that had not received the primary antibody was used as negative internal control. For immunohistochemical evaluation of the CD44 molecule we considered the membrane expression (Figure 1), calculating the percentage of positive epithelial cells with respect to the total number of cells encountered in 20 representative high power fields. We took no account of the intensity of the staining. We formed groups of tumors according to the percentage of stained cells (<25%, 25%-49%, 50%-74% and ≥75%) (8).

Table I. *Distribution and univariate influence of age and clinical variables on survival in tongue cancer (n=56).*

| Variable | n (%) | Cumulative survival proportion±se at 60 months | G: Global test[a] T: Tendency test[b] P: Paired comparisons[c] |
|---|---|---|---|
| Age (yr.) | | | |
| 37-49 | 16 (28.6) | 0.640±0.165 | G: $\chi^2$=0.02 (2 gl), $p$=0.990 |
| 50-69 | 26 (46.4) | 0.628±0.127 | T: $\chi^2_{tend.}$=0.00, $p$=0.959 |
| 70-87 | 14 (25.0) | 0.592±0.169 | |
| Clinical T | | | |
| T1 | 17 (30.4) | 0.941±0.057 | G: $\chi^2$=32.07 (3 gl), $p$<0.001 |
| T2 | 20 (35.7) | 0.829±0.119 | T: $\chi^2_{tend.}$=13.35, $p$<0.001 |
| T3 | 9 (16.1) | 0.000±0.000 | T1≠T3,T4; T2≠T3,T4 |
| T4 | 10 (17.9) | 0.267±0.207 | |
| Clinical N | | | |
| N0 | 33 (58.9) | 0.748±0.096 | G: $\chi^2$=2.50 (2 gl), $p$=0.287 |
| N1 | 11 (19.6) | 0.565±0.170 | T: $\chi^2_{tend.}$=2.10, $p$=0.147 |
| N2A (n=2) -N2B (n=10) | 12 (21.4) | 0.395±0.196 | |
| Clinical stage | | | |
| I | 14 (25.0) | 0.928±0.068 | G: $\chi^2$=10.61 (3 gl), $p$=0.014 |
| II | 14 (25.0) | 0.738±0.175 | T: $\chi^2_{tend.}$=9.64, $p$=0.002 |
| III | 9 (16.1) | 0.666±0.207 | P: I,II≠IV |
| IV | 19 (33.9) | 0.295±0.149 | |

a: Global log-rank test, for comparing survival curves for each category within a variable.
b: Tendency log-rank test (for variables with at least 3 ordered categories).
c: Only significantly ($p$<0.05) paired comparisons are denoted with the symbol "≠".

The statistical analysis was performed using SPSS for Windows, version 11.0 (SPSS Inc. Chicago, Illinois, USA). The association of CD44 with ordered parameters was estimated by Spearman's rank correlation. Survival times of the patients were gathered until 1998, distinguishing between complete (death for oral cancer) and incomplete (alive at last available date) times. No deaths for other reasons were recorded during the follow-up. The presence of tumor recurrence was also documented. The survival analysis was performed by means of the Kaplan-Meier method (17). A study was conducted of the association between the survival curves and different variables, grouping some categories to enable the analysis (Tables I and II), using global log-rank tests, or tendency log-rank tests for variables with three or more ordered categories. When there was statistical significance, pair-comparisons were carried out (17). Possible confounding factors were investigated using the Cox proportional hazards model (18). Potential variables were classified as described above, considering one of the categories as indicator and were included in the model according to their statistical significance ($p$<0.05 to enter and $p$>0.10 to exit the model, according to the likelihood ratio). During the construction of the model, variables that showed a correlation greater than 0.75 with previously included variables were excluded, to avoid colinearity effects.

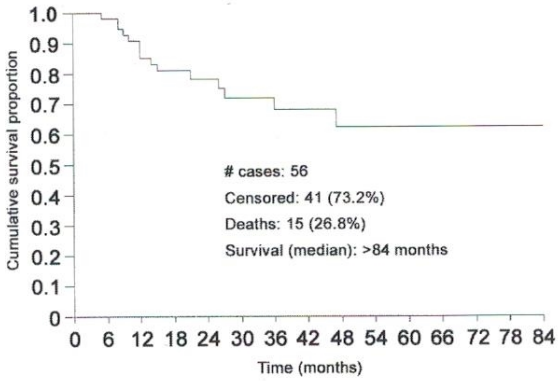


Figure 2. *Cumulative survival proportions in tongue cancer (n=56).*

## Results

The distribution of the clinical and pathologic variables is shown in the first three columns of Tables I and II, respectively. Twenty-five tumors, 44.6% of the total, presented CD44 expression in ≥75% of cells (Table II). No patient presented distant metastases (data not shown).

The mean 5-year survival rate for the whole series was 62.3% (standard error=8.8%). Figure 2 depicts the survival curve. Among the 15 non-survivors, cervical and local recurrence was presented by 7 and 5 patients, respectively, whereas among the 41 survivors it was presented by only 1 and 3 patients, respectively.

Analysis of the influence of age and clinical parameters on survival is shown in the last two columns of Table I. Clinical T and clinical stage were significantly associated with survival. Pathologic T, pathologic stage, tumor thickness and tumor CD44 expression were significantly associated with survival (Table II). In the present series, a greater tumor expression of CD44 was associated with a better tumor prognosis. Multivariate analysis showed that the parameter with greatest influence on survival was the CD44 expression. Once this variable was included in the model, no other variable entered.

## Discussion

Among a series of 56 patients with tongue cancer, 16 (28.6%) presented CD44 expression in at least 50% of the neoplastic cells, within the range reported by other authors (8,14,19-22). One of the most important aspects of CD44 expression in tongue cancer is its influence on the survival of the patients. In the present study, a reduction in CD44 expression had a negative effect on survival according to the univariate analysis ($p$<0.001) and was also the only variable to enter the Cox proportional hazards model. It seems

Table II. *Distribution and univariate influence of pathologic variables on survival in tongue cancer (n=56).*

| Variable | n (%) | Cumulative survival proportion±se at 60 months | G: Global test[a]<br>T: Tendency test[b]<br>P: Paired comparisons[c] |
|---|---|---|---|
| Pathologic T | | | |
| T1 | 17 (30.4) | 0.941±0.057 | G: $\chi^2$=37.17 (3 gl), $p$<0.001 |
| T2 | 22 (39.3) | 0.636±0.176 | T: $\chi^2_{tend.}$=21.35, $p$<0.001 |
| T3 | 9 (16.1) | 0.138±0.127 | P: T1≠T3,T4; T2≠T3,T4 |
| T4 | 8 (14.3) | 0.000±0.000 | |
| Pathologic N | | | |
| N0 | 35 (62.5) | 0.676±0.094 | G: $\chi^2$=0.53 (2 gl), $p$=0.769 |
| N1 | 13 (23.2) | 0.486±0.218 | |
| N2B(n=6)-N2C(n=1)-N3(n=1) | 8 (14.3) | 0.714±0.170 | T: $\chi^2_{tend.}$=0.38, $p$=0.536 |
| Pathologic stage | | | |
| I | 16 (28.6) | 0.937±0.060 | G: $\chi^2$=11.13 (3 gl), $p$=0.011 |
| II | 10 (17.9) | 0.500±0.250 | T: $\chi^2_{tend.}$=10.83, $p$=0.001 |
| III | 13 (23.2) | 0.448±0.204 | P: I≠III, IV |
| IV | 17 (30.4) | 0.278±0.208 | |
| P: IIIII,IV | | | |
| Degree of differentiation | | | |
| Well-differentiated | | | |
| Moderately-(n=15) or | 37 (68.5) | 0.656±0.106 | G: $\chi^2$=0.64 (1 gl), $p$=0.422 |
| Poorly-differentiated (n=2) | 17 (31.5) | 0.549±0.146 | |
| Unknown | 2 | | |
| Depth of invasion (mm.) | | | |
| 0-3 mm. | 10 (17.9) | 0.857±0.132 | G: $\chi^2$=6.28 (2 gl), $p$=0.043 |
| 4-7 mm. | 13 (23.2) | 0.555±0.248 | T: $\chi^2_{tend.}$=5.77, $p$=0.016 |
| ≥8 mm. | 33 (58.9) | 0.551±0.103 | P: 0-3 mm≠4-7 mm.,≥8 mm. |
| x=9.25 mm., s=5.54 mm. | | | |
| Surgical margin | | | |
| Free | 50 (90.9) | 0.653±0.093 | G: $\chi^2$=0.74 (1 gl), $p$=0.391 |
| Scant | 5 (9.1) | 0.600±0.219 | |
| Unknown | 1 | | |
| Tumor expression of CD44 | | | |
| <25% | 5 (8.9) | 0.000±0.000 | G: $\chi^2$=41.38 (3 gl), $p$<0.001 |
| 25-49% | 11 (19.6) | 0.180±0.150 | T: $\chi^2_{tend.}$=28.42, $p$<0.001 |
| 50-74% | 15 (26.8) | 0.933±0.064 | P: <25%,25-49%≠50-74%,≥75% |
| ≥75% | 25 (44.6) | 0.848±0.107 | |

a: Global log-rank test, for comparing survival curves for each category within a variable.
b: Tendency log-rank test (for variables with at least 3 ordered categories).
c: Only significantly ($p$<0.05) paired comparisons are denoted with the symbol "≠".

reasonable to conclude that the loss of CD44 expression releases cells from their adhesion to neighboring cells and favors their invasiveness, thereby behaving as a factor of poor prognosis. Our results support this hypothesis. However, the findings of other research groups have been contradictory. Some studies of oral cancer showed that a reduction in the expression of CD44v7 and CD44v9 had a negative influence on both survival and disease-free period (8,14). According to one of these studies (14), although the loss of expression affects only one tumor group, it increases the risk of death. This observation is also valid for the loss of expression of CD44s and CD44v6 in patients with laryngeal carcinoma, which is also significantly associated with a reduction in survival (23). In contrast, the relationship

between CD44 and survival is different in some other tumors. Stoll *et al.* (8) described three types or groups of tumors according to the relationship of the CD44 expression to survival. CD44 expression is correlated with poor prognosis in one group, which includes cancer of stomach, colon, breast, cervix, vulva and malignant lymphomas. In a second group, including ovarian and bronchial carcinomas, CD44 expression has no effect on survival, or the effect has not been clearly established, such as in renal carcinoma and malignant melanoma. In the third group, the loss of CD44 expression is correlated with a poor prognosis, such as in neuroblastoma, epidermal skin tumor and, as commented above, oral and laryngeal carcinomas. On the other hand, study of the literature (8) shows that many tumors have been classified into one or other group on the basis of only one or two studies. This seems clearly inadequate, especially given the variability in the method of CD44 expression assessment and in the CD44 isoforms studied. Aberrant, non-functional forms of CD44 may also have been produced by certain neoplastic cell types. It is important to bear in mind that the immunohistochemical expression of a protein does not necessarily imply function (24). *In vitro* studies (25) showed that CD44 expression in squamous cells was independent of function. Finally, it has also been proposed (21) that differences in the prognostic significance of CD44 may reflect a specifically regulated expression in each organ that differs according to the organ in question or even according to the pathologic type of the tumor.

A previous study by our group of the present series (5) demonstrated the importance of tumor thickness as an independent prognostic variable in tongue cancer. Tumors with a depth greater than 3 mm had a significantly worse prognosis than those that invaded less than 3mm. The present investigation showed that tumors with the greatest losses of CD44 expression presented significantly greater depths of invasion (Spearman correlation coefficient $r_s$=-0.37, $p$=0.005). As well as entering the Cox proportional hazards model, CD44 expression replaced the tumor thickness as the parameter that independently affected the survival. Our interpretation is that the loss of CD44 expression, by favoring cellular detachment, increases the invasiveness of the tumor and behaves as the original reason for the increased mortality in patients with deeper tumors (5). Some experimental observations support this interpretation. Mackay *et al.* (12) demonstrated an increase in invasion and cell migration of a malignant melanoma cell line after treatment with anti-CD44s monoclonal antibodies. Sato *et al.* (26) showed that the invasiveness of OSCC cell lines expressing high levels of CD44v9 (HSC-2 and HSC-3) significantly increased after treatment with anti-CD44v9 monoclonal antibodies, whereas the invasiveness of lines weakly expressing CD44v9 were not affected by the antibody treatment. Similar results were reported by Kanke *et al.* (21).

Our results constitute the first non-*in vitro* clinico-pathological evidence that the loss of CD44 expression increases the invasive capability of tongue carcinomas and that it behaves as a powerful marker of poor prognosis. Finally, the present study showed that the low expression of CD44 is also related to tumors with greater clinical ($r_s$=-0.51, $p$<0.001) and pathologic ($r_s$=-0.46, $p$<0.001) T, greater clinical ($r_s$=-0.52, $p$<0.001) and pathologic ($r_s$=-0.42, $p$<0.01) N and more advanced clinical ($r_s$=-0.56, $p$<0.001) and pathologic ($r_s$=-0.49, $p$<0.001) stage, all classically considered as markers of poor prognosis.

In conclusion, the reduced expression of CD44 behaves as a marker of poor tumor prognosis and it has demonstrated an independent influence on the survival of patients with tongue cancer.

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## References

1 Myers JN, Elkins T, Roberts D and Byers RM: Squamous cell carcinoma of the tongue in young adults: increasing incidence and factors that predict treatment outcomes. Otolaryngol Head Neck Surg *122*: 44-51, 2000.

2 Krause CJ, Lee JG and McCabe BF: Carcinoma of the oral cavity. Arch Otolaryngol *97*: 354-8, 1973.

3 Lee JG and Litton WB: Occult regional metastasis: carcinoma of the oral tongue. Laryngoscope *82*: 1273-81, 1972.

4 Hibbert J, Marks NJ, Winter PJ and Shaheen OH: Prognostic factors in oral squamous carcinoma and their relation to clinical staging. Clin Otolaryngol *8*: 197-203, 1983.

5 Gonzalez-Moles MA, Esteban F, Rodrvguez-Archilla A, Ruiz-Avila I and Gonzalez-Moles S: Importance of tumour thickness measurement in prognosis of tongue cancer. Oral Oncol *38*: 394-7, 2002.

6 Gonzalez-Moles MA, Galindo P and Gutiurrez-Fernundez J *et al*: P53 protein expression in oral squamous cell carcinoma. Survival analysis. Anticancer Res *21*: 2889-94, 2001.

7 Gonzales-Moles MA, Rodrvguez-Archilla A, Ruiz-Avila I, Martvnez AB, Morales-Garcva P and Gonzalez-Moles S: p16 expression in squamous carcinomas of the tongue. Onkologie *25*: 433-6, 2002.

8 Stoll C, Baretton G, Soost F, Terpe HJ, Domide P and Lohrs U: Prognostic importance of the expression of CD44 splice variants in oral squamous cell carcinomas. Oral Oncol 35: 484-9, 1999.

9 Bryne M: Is the invasive front of an oral carcinoma the most important area for prognostication? Oral Dis *4*: 70-7, 1998.

10 Mori S, Nose M and Morikawa H *et al*: A novel evaluation system of metastatic potential of oral squamous cell carcinoma according to the histopathological and histochemical grading. Oral Oncol *34*: 549-57, 1998.

11 Borland G, Ross JA and Guy K: Forms and functions of CD44. Immunology *93*: 139-48, 1998.

12 Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H and Gunthert U: Expression and modulation of CD44 variant isoforms in humans. J Cell Biol *124*: 71-82, 1994.
13 Sneath RJ and Mangham DC: The normal structure and function of CD44 and its role in neoplasia. Mol Pathol *51*: 191-200, 1998.
14 Sato S, Miyauchi M and Takekoshi T *et al*: Reduced expression of CD44 variant 9 is related to lymph node metastasis and poor survival in squamous cell carcinoma of tongue. Oral Oncol *36*: 545-9, 2000.
15 Spiessl B: International Union Against Cancer. TNM Atlas Illustrated Guide to the TNM, pTNM Classification of Malignant Tumors. 3rd ed. 2nd revision. Berlin, New York: Springer-Verlag, 1992.
16 Arcangeli G, Mauro F, Nervi C and Starace G: A critical appraisal of the usefulness of some biological parameters in predicting tumour radiation response of human head and neck cancer. Br J Cancer Suppl *41*: 39S-44S, 1980.
17 Domınech JM and Sarria A: Analisis de Supervivencia. Barcelona: Editorial Graficas Signo, SA, 1993.
18 Domınech JM and Sarria A: Modelo de Regresion de Cox. Barcelona: Editorial Graficas Signo SA, 1993.
19 Hirvikoski P, Tammi R and Kumpulainen E *et al*: Irregular expression of hyaluronan and its CD44 receptor is associated with metastatic phenotype in laryngeal squamous cell carcinoma. Virchows Arch *434*: 37-44, 1999.
20 Ioachim E, Assimakopoulos D, Goussia AC, Peschos D, Skevas A and Agnantis NJ: Glycoprotein CD44 expression in benign, premalignant and malignant epithelial lesions of the larynx: an immunohistochemical study including correlation with Rb, p53, Ki-67 and PCNA. Histol Histopathol *14*: 1113-8, 1999.
21 Kanke M, Fujii M and Kameyama K *et al*: Role of CD44 variant exon 6 in invasion of head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg *126*: 1217-23, 2000.
22 Fonseca I, Pereira T, Rosa-Santos J and Soares J: Expression of CD44 isoforms in squamous cell carcinoma of the border of the tongue: A correlation with histological grade, pattern of stromal invasion and cell differentiation. J Surg Oncol *76*: 115-20, 2001.
23 Spafford MF, Koeppe J, Pan Z, Archer PG, Meyers AD and Franklin WA: Correlation of tumor markers p53, bcl-2, CD34, CD44H, CD44v6 and Ki-67 with survival and metastasis in laryngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg *122*: 627-32, 1996.
24 Helliwell TR: Molecular markers of metastasis in squamous carcinomas. J Pathol *194*: 289-93, 2001.
25 Bruno S, Fabbi M and Tiso M *et al*: Cell activation *via* CD44 occurs in advanced stages of squamous cell carcinogenesis. Carcinogenesis *21*: 893-900, 2000.
26 Sato S, Miyauchi M and Ogawa I *et al*: Inhibition of CD44v9 upregulates the invasion ability of oral squamous cell carcinoma cells. Oral Oncol *39*: 27-30, 2003.

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