Intense CD44 Expression Is a Negative Prognostic Factor in Tonsillar and Base of Tongue Cancer

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Abstract. *Background: Patients with tonsillar and base of tongue cancer, which are human papillomavirus (HPV) positive, have a better clinical outcome than those with HPV-negative tumors. The identification of additional predictive markers for response to therapy could still be of great use. Materials and Methods: Tumor markers CD44, p16, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), E-cadherin, cyclooxygenase-2 (COX 2), Ki-67, and p27 were analyzed by immunochemistry, and HPV status was tested by polymerase chain reaction (PCR) in tumors from 73 patients and correlated to survival. Results: High intensity CD44 staining (p=0.006) and high EGFR expression (p=0.026) were indicators of poor prognosis, while high p16 expression (p=0.021) and younger age (p=0.002) were positive prognostic markers for disease-specific survival. Furthermore, staining of CD44 (p=0.026) and age (p=0.002) were shown to be strong prognostic markers in multivariate analysis, which should be evaluated further for possible use in clinical practice.*

In Sweden, head and neck cancer comprises 3-4% of all cancer cases, with the most common sub-group being tonsillar cancer (1). Main risk factors for head and neck cancer are alcohol consumption and smoking (2), but since 2007, the International Agency for Research on Cancer (IARC) has acknowledged human papillomavirus (HPV) as a risk factor for oropharyngeal squamous cell cancer (3).

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The incidence of oropharyngeal cancer is increasing in many countries, including Sweden (1, 4-8), despite a decrease in smoking prevalence, and HPV infection has been suggested to be responsible for this increase (1, 9-14). There is also strong evidence for presence of HPV being a positive prognostic factor in oropharyngeal cancer, and patients with HPV-positive tumors, especially non-smokers, have a much better disease-specific survival than patients with HPV-negative tumors (9, 12, 13, 15-21). However, the biological reason for this remains to be elucidated, and why the outcome for some patients with HPV-positive tumors is poor is still unknown.

Recently, treatment for oropharyngeal cancer, including tonsillar and base of tongue cancer, the two dominant oropharyngeal cancer sub-groups and the two with the strongest association with HPV (9-14), has been intensified. Patients are now often treated with induction chemotherapy, intensified hyperfractionated radiotherapy, and in some cases epidermal growth factor receptor (EGFR) inhibitors, which results in additional side-effects. To identify which patients may benefit from and really need this intensified treatment demands further analysis. In this respect, the identification of additional prognostic and predictive markers with regard to response to treatment of HPV-positive and HPV-negative oropharyngeal cancer would be of great use.

For this purpose, we assessed HPV status and expression of CD44, p16, EGFR, vascular endothelial growth factor (VEGF), E-cadherin, cyclooxygenase-2 (COX 2), Ki-67, and p27 for correlation to clinical outcome. There are specific reasons for selecting these molecular markers. High expression of the tumor suppressor gene *p16* has previously been shown to correlate to the presence of HPV and a better disease specific survival in oropharyngeal cancer (22-26). Moreover, p16 has been suggested as a surrogate marker for HPV in clinical practice for evaluating the tumor as HPV-positive and indicating HPV as being functionally active, particularly since the presence of HPV and p16 is not always found simultaneously (24, 27). P27, similarly to p16, is a cell cycle regulator and regulates cell proliferation, motility and

apoptosis (28). CD44 is the major hyaluronan receptor important for homing and settling of metastasizing tumor cells (29), and could thus be a negative factor for prognosis and has been used in some head and neck cancer studies, but not specifically for tonsillar and base of tongue cancer (30, 31). Expression of CD44 includes several isoforms, but here only the presence of the standard variant was investigated. Expression of EGFR has been studied in many types of cancer, including that of the head and neck, and an inverse correlation of EGFR expression with HPV as well as with survival, has been reported (27, 32, 33). High expression of VEGF, Ki-67 and expression of COX-2 have been associated with poor prognosis in some sub-types of head and neck cancer (34-38), but none of these markers have, to our knowledge, been studied together with HPV status in tonsillar and base of tongue cancer. Finally, expression of E-cadherin has been suggested as a predictor of response to radiotherapy in head and neck cancer as a group (39), but has not been studied specifically for tonsillar and base of tongue cancer. Notably, studying the expression of COX-2, EGFR and VEGF are of particular interest since treatment targeted to these is available.

Here, we examined the incidence of tonsillar and base of tongue cancer, and the prevalence of HPV in these tumors during the period of 1970-2007, in the rural sparsely populated county of Dalarna in Sweden. In parallel, we analyzed and compared the expression of tumor markers CD44, p16, EGFR, VEGF, E-cadherin, COX-2, Ki-67, and p27 in tonsillar and base of tongue cancer and correlated their expression to disease-specific survival.

Materials and Methods

Patients and materials. Between 1970-2007, 102 patients were identified with squamous cell carcinoma of the tonsil and base of tongue in the County of Dalarna through the Swedish Cancer Registry, to which all cancer cases are reported (40). The study was conducted with permission 2009/256 from The Regional Ethical Review Board in Uppsala, and only three patients did not consent to participate in the study. After re-review of the patient files, 12 patients were excluded, and 87/99 patients with correct diagnoses remained. Of the 12 patients excluded due to incorrect diagnoses 11 patients had a squamous cell carcinoma at another anatomical location (most frequently a tumor originating from the part of the tongue that is in the oral cavity), and one patient had another histological subtype (sarcoma) than squamous cell carcinoma. From the 87 patients with correct diagnoses, 79 paraffin-embedded pretreatment diagnostic biopsies were obtained, of which 6 were excluded due to limited material, hence 73 patients were included in the study (Table I). Notably, no difference in age, gender, tumor localization and grade was found when comparing the 73 patients included in the study with the 14 excluded patients with correct diagnosis, but with unavailable or insufficient biopsy material (data not shown).

Available biopsies were successfully analyzed for expression of biological tumor markers by immunohistochemistry (IHC) for all 73 patients and for the presence by HPV by PCR in 56 cases.

Table I. *Patient and tumor characteristics*.

Patient and tumor characteristics	
Patients included in the study	73
Age at diagnosis (years)	
Median	59
Mean	61
Gender (n=73)	
Male	59
Female	14
Tumor localization (n=73)	
Tonsil	55
Base of tongue	18
TNM stage (n=73)	
I	0
II	5
III	7
IV	48
Unknown	13
Histopathological grade (n=73)	
Well differentiated	3
Moderately differentiated	36
Poorly differentiated	31
Unknown	3
Primary treatment (n=73)	
Curative	62
Palliative	4
Unknown	7
Outcome (n=62)	
Died free of disease	24
Died of disease	22
Alive, no evidence of disease	17
Follow up time all patients (months)	
Median	37
Mean	55
Follow up time, excluding patients dead of disease (months)	
Median	77
Mean	78

Clinical data was collected from patient files, including TNM stage, tumor localization, relapse, treatment and cause of death. From the 73 patients included in this study, survival analysis was performed on data for the 62 patients, who were treated with intention to cure, by pretreatment radiotherapy, in most cases, followed by surgery (Table I). In four cases the patients only received palliative treatment and in seven cases no data were available (Table I).

Detection of HPV DNA by polymerase chain reaction (PCR). A total of three 10 μm slices from the paraffin embedded biopsies were cut and DNA was extracted using High Pure RNA kit (Roche Diagnostics, Stockholm, Sweden) according to the manufacturers' instructions excluding DNAse treatment. A negative control between each sample was included to detect and avoid cross contamination. High-risk HPV DNA was detected using the PCR method with general primers Gp5+/6+ (41) and CpI/IIG (42), targeting the L1 and E1 region of the genome respectively, and the detailed conditions of these two analyses, as well as HPV typing using HPV16 type-specific primers or Multiplex Luminex have been described previously (17, 43). This approach detects almost all high-

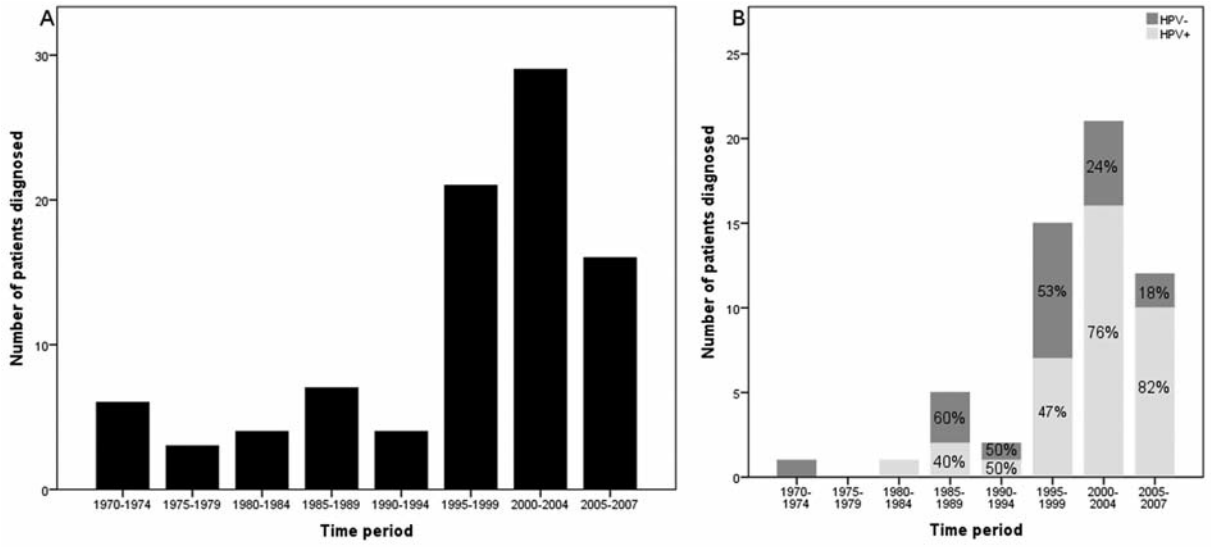

Figure 1. *The number of patients diagnosed with tonsillar and base of tongue cancer is increasing in Dalarna, and in total 73 patients shown here (A). In parallel, there is also an increase in the percentage of HPV positive tumors over time, when illustrating data for patients where HPV status was obtained (B). Notably, the rightmost bar for both graphs includes cases diagnosed only within a 3-year period.*

risk HPV types commonly found in both oropharyngeal and cervical cancer. All HPV-negative samples were run in an S14 PCR to verify presence of amplifiable DNA, as previously described (44).

Immunohistochemistry (IHC) of CD44, p16, EGFR, VEGF, E-cadherin, COX-2, Ki-67, and p27. One 3 μm section from each of the paraffin blocks were reviewed by a senior pathologist and a representative area of the tumor (containing at least 70% tumor tissue) was marked for tissue microarray (TMA). Punch biopsies were taken from the blocks and joined together into a TMA paraffin block, including controls. IHC was then performed with a Ventana Benchmark XT (Roche Diagnostics GmbH, Mannheim, Germany). The antigen retrieval was performed for all primary antibodies by overnight incubation in 0.1 citric acid, pH 7.2, at 65˚C. The primary antibodies used were p16 (clone INK4A dilution 1:25, PharMingen, San Diego, California, USA); COX-2 (clone SP 21, dilution 1:50, NeoMarkers, Freemont, USA); VEGF (polyclonal, dilution 1:50, Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA); Ki-67 (clone MIB-1, dilution 1:100), EGFR (clone E30, dilution 1:50), E-cadherin (clone NCH-38, dilution 1:25), CD44 (clone DF1485, dilution 1:50), and p27 (clone SX53G8, dilution 1:25, all DAKO, Glostrup, Denmark). Biotinylated secondary goat anti-mouse antibody was used for the detection system and streptavidin-horseradish peroxidase conjugate was used for visualization of diaminobenzidine solution. A solution of streptavidin was used to block endogenous biotin activity. All procedures were performed at the Department of Pathology and Clinical Cytology, Falun Central Hospital.

A senior pathologist, blinded for all clinical data, evaluated the expression of the molecular markers. Both the intensity of the staining and the percentage of positively stained cells were evaluated. The intensity of all cells that had a positive staining was estimated on a semi-quantitative four-grade scale from 0 to 3+ (equal to absent, mild, moderate and severe, where 0 being absent), and the percentage of positive cells was also evaluated on a four-grade semi-quantitative scale of 0, 1-10%, 11-49% and >50% positive cells (45).

Statistical analysis. All significance testing was performed at the 0.05 level. The association between ordinal variables was tested using a Chi-square test or Fisher's exact test where appropriate. All molecular markers and HPV status were correlated to disease-specific survival, illustrated in a Kaplan-Meier graph, and a log-rank test was used for comparison between the groups. All markers with significant results in the univariate analysis, as well as factors generally regarded to possibly influence survival, *e.g.* age, TNM stage, grade of differentiation, and tumor localization, were then further tested in a Cox regression multivariate analysis to evaluate factors influencing the mortality risk.

Results

Characterization of patients with tonsillar and base of tongue cancer and their tumors. The number of patients diagnosed with tonsillar and base of tongue cancer in the county of Dalarna, with a population of around 250,000, has increased, and the data for each five-year period between 1970-2007 are illustrated in Figure 1A. In parallel in Figure 1B, data are illustrated for the 56 patients, for whom the HPV status of the tumors was analyzed successfully.

Detailed characteristics of the 73 patients with tonsillar and base of tongue cancer, and their tumors are presented in Table I, however, reliable data on smoking habits could not be obtained from patients' files. The median follow-up time for all patients included in the study was 37 months, with a range of 0-178 months; for the patients not dying from their disease, the median follow-up time was 78 months with a range of 2-178 months. Age was found to be a prognostic factor for survival in the multivariate analysis (Table II). There were no differences in survival however, when

comparing tumor localization (*i.e.* between tonsillar and base of tongue cancer), grade of differentiation, TNM stage, regional lymph node status and sex in the univariate (data not shown) and multivariate analyses (Table II).

Expression of molecular markers, CD44, p16, EGFR, VEGF, E-cadherin, COX-2, Ki-67 and p27 and their correlation to survival. It was possible to evaluate the intensity and expression of CD44, p16, EGFR, VEGF, E-cadherin, COX-2, Ki-67, and p27 for 73 cases, and appropriate controls confirmed that the analysis worked properly. All molecular markers were evaluated using a Kaplan-Meier plot that was dichotomized using the median as a cut-off level, resulting in two groups of similar size. These two groups were then compared both in univariate and multivariate analysis, as described below. Survival analyses regarding IHC were performed for the 62 patients treated with intention to cure and who could be evaluated further. The expression of three molecular markers, CD44, p16 and EGFR, were found to correlate with prognosis in a univariate analysis, while the intensity and expression of VEGF, E-cadherin, COX-2, Ki-67, and p27 were not significantly correlated to survival in this study.

The results when evaluating CD44 intensity before and after dichotomization are shown in Figure 2A and B, respectively. There was a significant correlation between high CD44 intensity staining and poor survival (log-rank test p=0.006) as shown in Figure 2B. However, correlation between a high expression and survival was not statistically significant after dichotomization with a cut-off level at staining of 10% cells (log-rank test p=0.162). A high intensity of CD44 was also shown to be a negative prognostic factor in a Cox proportional hazard regression multivariate analysis including age at diagnosis, TNM stage, grade, tumor location and sex (p=0.012; other data not shown).

High expression of EGFR, but not high intensity of the staining, also correlated with survival (Figure 2C and D). Different grades of expression were dichotomized with a cut-off level at staining of 10% cells and when evaluated in a Kaplan-Meier plot and compared in a log-rank test, a high expression of EGFR correlated with a worse survival (p=0.026), illustrated in Figure 2D.

Both a high expression of p16 and a high intensity of staining were shown to be positive prognostic factors when dichotomizing with a cut-off level at 10% (log-rank test, p=0.021) as shown in Figure 3A, or when comparing 0 and 1+ with 2+ and 3+ (p=0.041). In a multivariate analysis, including age at diagnosis, TNM stage, grade, tumor location and sex, a high expression of p16 remained a positive prognostic factor for survival (p=0.018; other data not shown), but intensity of p16 staining did not (p=0.098, other data not shown).

When analyzing survival with the log-rank test, none of the other markers reached statistical significance when comparing intensity and expression, including VEGF (p=0.174 and p=0.345), E-cadherin (p=0.355 and p=0.395), COX-2 (p=0.944 and p=0.599), Ki-67 (p=0.628 and p=0.145) and p27 (p=0.690 and p=0.471).

Finally, CD44, p16 and EGFR were also included in the multivariate analyses together with age, TNM stage, grade, tumor location and sex. In this analysis, only intensity of CD44 remained a statistically significant prognostic marker (p=0.026), while expression of p16 and EGFR failed to show a statistically significant correlation in the multivariate analysis (Table II).

Table II. *Multivariate analysis including CD44, p16 and EGFR.*

	HR	(95% CI)	p-Value
CD44 intensity, high *vs.* low	13.005	(1.353-124.983)	0.026
p16 expression, high *vs.* low	0.491	(0.190-1.269)	0.142
EGFR expression, high *vs.* low	2.953	(0.868-10.052)	0.061
Age at diagnosis	1.094	(1.034-1.158)	0.002
TNM stage	0.799	(0.563-1.135)	0.211
Grade[a]	1.191	(0.540-2.629)	0.665
Tumor localization[b]	0.960	(0.282-3.265)	0.948
Gender	2.937	(0.950-9.080)	0.061

HR: Hazard ratio. CI: Confidence interval; [a]Grade of differentiation. [b]Tonsillar cancer and base of tongue cancer.

HPV status over time and its correlation with clinical features. HPV status was obtained for 56 out of the 73 tumors, since 17 cases could not be analyzed due to there being a limited amount of material. In total, 36 (64%) were HPV-positive, all of which were HPV type 16, and 20 (36%) were HPV-negative. In parallel to the increased incidence of tonsillar and base of tongue cancer, there was a significant increase in the proportion of patients with HPV-positive tumors when comparing the 24 patients diagnosed 1970-1999 with the 32 patients diagnosed in 2000-2007 (p=0.013, Figure 1B). In addition, patients with HPV-positive tumors (median age=58 years) were significantly younger than those with HPV-negative tumors (median age=67 years, p=0.044, Mann-Whitney test). Survival analyses, including a Kaplan-Meier plot and log-rank test, in relation to HPV status was obtained for 46 out of the 56 patients, when excluding the patients given palliative treatment (n=4), and those (n=6) where treatment data was not possible to obtain. There was an obvious tendency for patients with HPV-positive tumors to have a better disease-specific survival, but this did not reach statistical significance (p=0.23, Figure 3B). Therefore, HPV status is not included in the multivariate analyses presented in Table II. However, if included, CD44 and age remained strong and significant prognostic factors (p=0.004 and p=0.002, respectively),

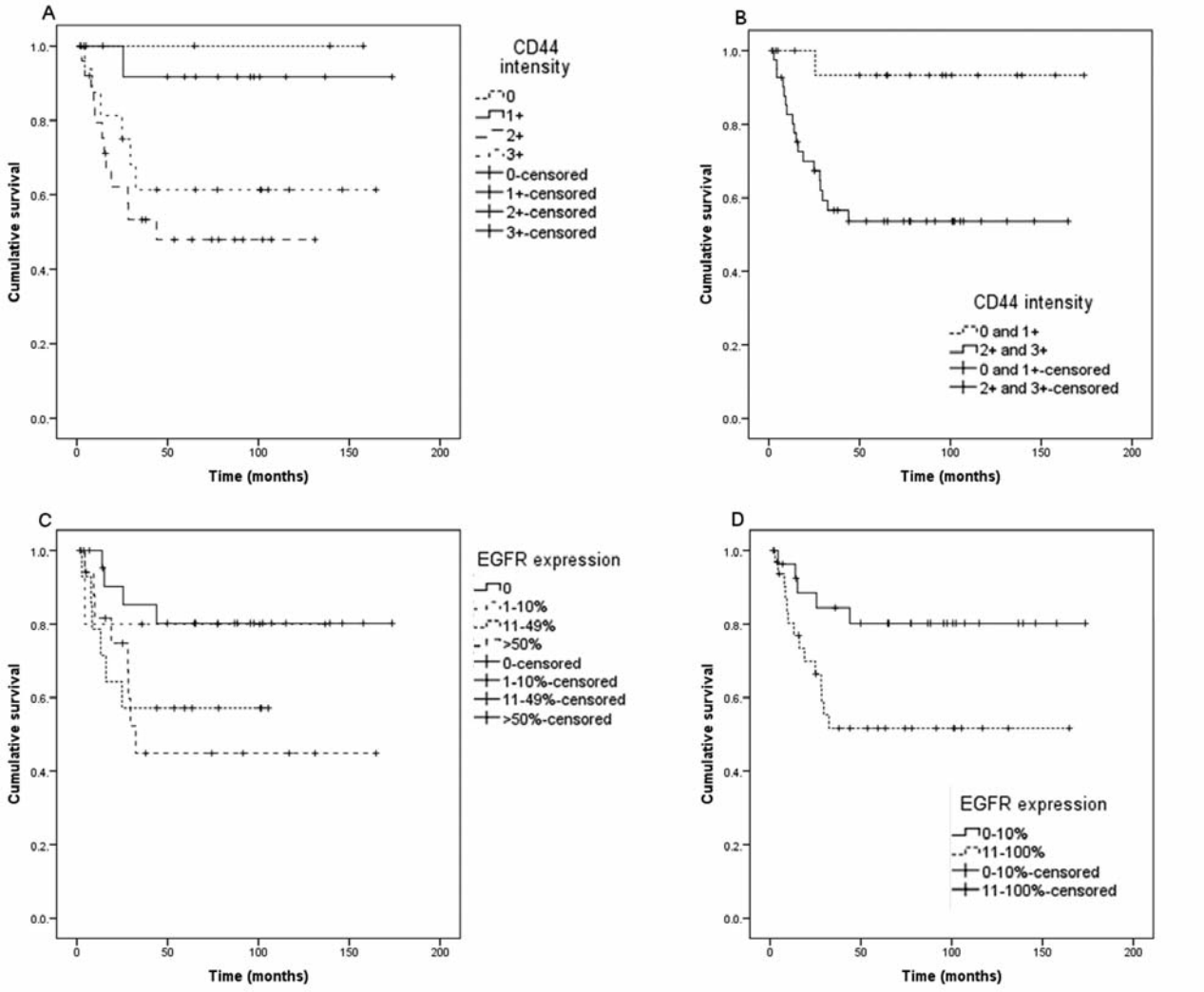

Figure 2. *Kaplan-Meier graphs of survival for 62 patients, depending on the intensity of expression of CD44 and expression of EGFR evaluated on a four grade semi-quantitative scale. Survival rate is shown for all four groups for CD44 (A), and for EGFR (C), and after dichotomization (B and D, respectively). A highly significant association for both CD44 intensity and EGFR expression with survival was found (log-rank test, p=0.006 and p=0.026, respectively).*

whereas expression of p16 and EGFR did not (detailed data from the analysis not shown).

Correlation between HPV status and intensity and expression of tumor markers. The correlation between HPV status and the expression and intensity of the IHC analysis for p16, CD44, EGFR, COX-2, Ki-67, VEGF, E-cadherin, and p27 was evaluated for all 56 patients where HPV status had been obtained (Table III). A significant correlation between HPV status and both expression and intensity of p16 was found (*p*-values 0.026 and 0.016, respectively, Chi-square test, and for details see Table III). Expression and intensity of CD44, EGFR, COX-2, Ki-67, VEGF, E-cadherin, and p27 IHC was not correlated to HPV status (see Table III).

Discussion

In this study, between the years 1970-2007, we demonstrated a parallel increase in the incidence of tonsillar and base of tongue cancer and the proportion of HPV-positive tumors in patients from the rural area of Dalarna, Sweden. In addition, CD44, p16, EGFR, VEGF, E-cadherin, COX2, Ki-67 and p27 staining by IHC and HPV status analyzed by PCR were evaluated as prognostic markers for survival for patients with tonsillar and base of tongue cancer. We observed that high intensity CD44 staining and EGFR overexpression were correlated to poor prognosis, while p16 overexpression was correlated to a better prognosis. High intensity staining for CD44 gave better statistical significance than p16

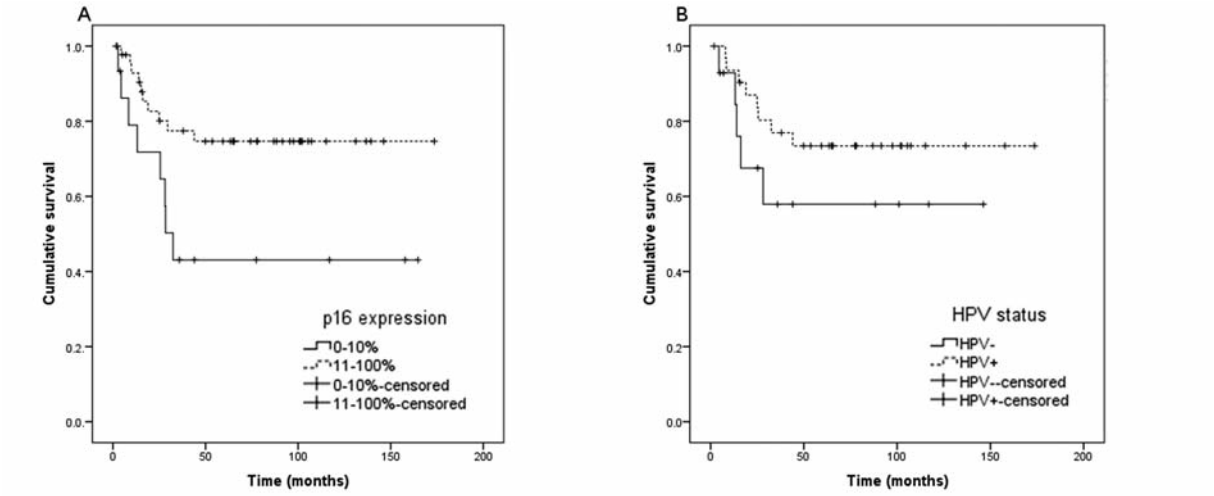

Figure 3. *A: Kaplan-Meier graph of survival for 62 patients depending on the percentage of tumor cells expressing p16, using a cut-off level at 10% (log-rank test, p=0.021). B: Kaplan-Meier graph of survival for 46 patients depending on HPV status, showing that patients with HPV-positive tumors had a tendency to have a better survival than those with HPV-negative tumors (log-rank test, p=0.23).*

Table III. *Correlation of tumor markers with HPV status.*

Tumor marker	HPV status	Cells with positive expression (n=56)[1]				Intensity of staining (n=56)			
		0	1-10%	11-49%	≥50%	0	1+	2+	3+
p16	Pos	6	0	6	23	6	7	11	12
	Neg	3	6	2	9	3	9	2	6
COX-2	Pos	10	14	9	3	10	16	6	4
	Neg	6	3	6	5	6	6	4	4
Ki-67	Pos	0	3	13	20	0	7	16	13
	Neg	0	2	8	10	0	7	9	4
EGFR	Pos	16	5	10	5	16	14	5	1
	Neg	6	3	5	6	6	7	4	3
VEGF	Pos	0	2	2	32	0	7	18	11
	Neg	0	1	1	18	0	3	13	4
E-Cadherin	Pos	0	0	0	36	0	3	7	26
	Neg	0	1	1	18	0	3	6	11
CD44	Pos	2	7	8	19	2	9	16	9
	Neg	0	3	4	13	0	4	7	9
p27	Pos	4	15	6	10	4	22	7	2
	Neg	5	10	2	3	5	12	3	0

[1]When evaluating cells with positive p16 expression, the sample from one patient could not be evaluated due to technical reasons.

overexpression in this study, which has not been reported previously. HPV status showed a tendency for being a positive prognostic marker, but here the number of cases was more limited compared to those included in the IHC analysis and statistical significance was not obtained.

The fact that both the incidence of tonsillar and base of tongue cancer and the proportion of HPV-positive tumors is increasing in a rural area in Sweden has not been demonstrated previously, but is perfectly in line with the trend in a large city, namely Stockholm, in the same country (10, 11). It also emphasizes the need to find better predictive factors for future individualized therapy of this increasing group of patients.

Here, tonsillar and base of tongue cancer biopsies from the 62 patients treated with a curative intention were evaluated for intensity and expression of the standard variant of CD44, and high intensity CD44 staining showed a statistically significant association with worse survival in a multivariate analysis. The prognostic value of CD44 expression has been suggested in one previous head and neck

cancer study focusing on mesopharyngeal cancer, however, in that report there are no details on sub-sites (31). Here, only sub-sites associated with HPV were included and the intensity of CD44 staining was shown to be a stronger prognostic marker than the percentage of CD44-positive cells and this was still valid in multivariate analysis including other important known prognostic factors, such as p16, EGFR and age. Our results somewhat contrast to the findings of two earlier studies (30, 46), but are in line with a third, more recent, publication (47). In one of the earlier studies, only 9 out of the 99 studied tumors were of oropharyngeal origin, and different splice variants of CD44 were evaluated. In that study, reduced CD44 expression was found to correlate with shorter survival (46). Moreover, in that report, only the percentage of cells expressing CD44, and not the intensity of the staining, was evaluated. In the other earlier study, 6 out of the 25 tumors studied were of oropharyngeal origin and an increased expression of CD44 was correlated with longer survival, but again, only the percentage of expression and not the intensity of staining was assessed (30). Nevertheless, since a sub-group analysis was not performed in either of these reports and there was a very limited number of patients with oropharyngeal cancer, it is very difficult to draw definite conclusions or perform comparisons between the studies regarding this specific patient group. In the more recent publication, the predictive value of CD44 staining was studied in laryngeal cancer (47). The results in that study are in line with those we report here, and CD44 was also found to correlate with a higher rate of recurrence in patients treated with radiotherapy (47).

Together these reports highlight that tumors, especially in the head and neck region, should be studied separately for each biological sub-site and not be studied as one single group. In addition, examining the intensity of the staining may also be of importance.

In concordance with previous reports, in our study p16 staining was also a positive prognostic marker for clinical outcome in patients treated with intention to cure (24-26). It has also been suggested that HPV-positive cancer expressing p16 represents a sub-group with evidence of biologically active HPV infection, and patients within this sub-group have an even better prognosis than those with tumors only positive for HPV (25). Here however, despite a statistically significant correlation between p16 expression and HPV status, there was only a tendency for HPV to be a prognostic factor in patients with tonsillar and base of tongue cancer. The reason statistical significance for p16 (n=62), but not HPV (n=46), was reached was most likely due to the more limited number of patients in the latter group, where both patient data including treatment with intent to cure and HPV status was obtained. However, our findings are still in concordance with previous studies implying HPV to be a positive prognostic factor (9, 16-21, 25, 26).

In this study, we found an inverse correlation between EGFR expression and survival, which is perfectly in line with other studies (27, 32, 33). An inverse correlation between HPV status and expression of EGFR is also frequently reported (27, 32, 33), but this we did not confirm here, most likely due to the limited number of cases where HPV status was analyzed.

Age was also found to correlate with prognosis, with younger patients having a better clinical outcome, which was expected (16). In addition, there was also a statistically significant difference in median age, with patients with HPV-positive tumors being younger than those with HPV-negative tumors, a finding which has been reported previously (10).

When including CD44, p16 and EGFR in a multivariate analysis, including other known prognostic factors such as age at diagnosis, TNM stage, grade, sex and tumor localization, high intensity CD44 staining still remained a significant prognostic factor, whereas p16 and EGFR were then no longer significantly associated with survival. This suggests that CD44 may be a strong prognostic marker, independently of EGFR and p16 expression. Nevertheless, this is a pilot study indicating a prognostic value of high intensity CD44 staining in tonsillar and base of tongue cancer, and a follow-up of study with a larger number of cases, including more detailed data regarding different treatment modalities, would be valuable.

One limitation of our study is the restricted number of cases, and this may contribute to the fact that we did not identify VEGF, Ki-67, COX-2, E-cahderin or p27 as prognostic factors here. Concordantly to this report however, a recent study including 111 oral and oropharyngeal cancer patients, with only 17 cases of the latter, did not show that p27 or Ki-67 expression was correlated to treatment response and survival after treatment with preoperative chemoradiation (48). Nevertheless, high expression of Ki-67 has been associated with poor prognosis in laryngeal cancer (34) and in cancer of the oral cavity and tongue (38). Furthermore, both high expression of VEGF and COX-2 expression have been associated with worse survival in head and neck cancer in general (35, 36), and could thus still be useful as prognostic markers for some head and neck sub-sites. In fact, low COX-2 expression has been correlated with poor prognosis more specifically in oral tongue cancer (37). Likewise, the expression of E-cadherin has been suggested as a predictor of response to radiotherapy in head and neck cancer as a group (39), and could thus be a useful marker for some sub-sites despite negative findings in this study.

In conclusion, in this study, intense staining of CD44 was shown to be a negative prognostic marker in tonsillar and base of tongue cancer, irrespective of HPV status, and this correlation should be evaluated further in larger patient groups before its possible use in clinical practice. It was also demonstrated that p16, EGFR and also, presumably, the presence of HPV are prognostic factors for survival in

tonsillar and base of tongue cancer in line with previous reports. Finally, similar to our previous findings in a Stockholm cohort, we find both an increased incidence of tonsillar and base of tongue cancer and an increase in the proportion of HPV-positive patients in a rural area in Sweden, suggesting that this trend is not limited to urban areas.

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