In HPV-negative oropharyngeal squamous cell carcinoma, elevated toll-like receptor 2 immunoexpression may increase the risk of disease-specific mortality

Anna Kaisa Kylmä,[a] Lauri Jouhi,[b] Hesham Mohamed,[a] Reija Randén-Brady,[a] Antti Mäkitie,[b,c,d] Timo Atula,[b] Caj Haglund,[e,f] Timo Sorsa,[g,h] Jaana Hagström[a,f,i]

Affiliations

[a]Department of Pathology, University of Helsinki, HUSLAB and Helsinki University Hospital, P. O. Box 21, 00014 University of Helsinki Helsinki, Finland

[b]Department of Otorhinolaryngology - Head and Neck Surgery, University of Helsinki and Helsinki University Hospital, P. O. Box 263, 00029 HUS Helsinki, Finland

[c]Division of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and Technology, Karolinska Institutet and Karolinska Hospital, Stockholm, Sweden

[d]Research Program in Systems Oncology, Faculty of Medicine, University of Helsinki, Helsinki, Finland

[e]Department of Surgery, University of Helsinki and Helsinki University Hospital, P. O. Box 20, 00014 University of Helsinki, Helsinki, Finland

[f]Research Programs Unit, Translational Cancer Medicine, University of Helsinki, P. O. Box 63, 00014 University of Helsinki, Helsinki, Finland

[g]Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Central Hospital, P. O. Box 41, 00014 University of Helsinki, Helsinki, Finland

[h]Division of Periodontology, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden

[i]Department of Oral Pathology and Radiology, Institute of Dentistry, Faculty of Medicine, University of Turku, Turku, Finland

Corresponding author

Anna Kaisa Kylmä, MS, BDS

Department of Pathology, University of Helsinki, HUSLAB and Helsinki University Hospital,

P.O. Box 21, FI-00014 University of Helsinki, Helsinki, Finland, Tel +358-50 4015651

Fax +358-9-47175010, E-mail address: anna.kylma@helsinki.fi

Word count

3082 words

Conflict of interest

None declared.

Abstract

Objectives: In oropharyngeal squamous cell carcinoma (OPSCC), toll-like receptors (TLR) 5 and 7 associate with the tumor's human papilloma virus (HPV) status [1]. TLR 2, on the other hand, has been linked to head and neck squamous cell carcinoma (HNSCC), and to oral carcinogenesis [2,3]. Here we investigated the presence of TLR 2 and 4 in HPV-positive and HPV-negative OPSCC, and their relationship to opportunistic oral pathogen Treponema denticola chymotrypsin-like protease (Td-CTLP) immunoexpression, clinical parameters, and patient outcome.

Materials and methods: Clinicopathological data of 198 unselected consecutive OPSCC patients came from hospital registries. Immunoexpression of TLRs 2 and 4 we evaluated by immunohistochemistry, and earlier in this patient series we studied immunoexpression of Td-CTLP and HPV DNA, HPV mRNA, and p16 status.

Results: Immunoexpression of both TLRs 2 and 4 showed a significant association with HPV-status. Strong expression was associated with HPV-positivity and mild expression with HPV-negativity. Patients with strong TLR 2 immunoexpression in the HPV negative subgroup had significantly poorer 5-year DSS (58%) than did patients with mild TLR 2 expression (77%), and strong TLR 2 immunoexpression remained as an independent factor linked to increased disease mortality in the multivariable setting (P=0.019). No association existed between TLR 2 or 4 and Td-CTLP expression.

Conclusion: Our results support the role of TLR 2 receptor as a possible target for development of therapeutics as earlier proposed [2]. The involvement of Td and other oral pathogens in carcinogenesis of OPSCC, remains open and calls for further study.

Keywords

Oropharyngeal squamous cell carcinoma, human papillomavirus, toll-like receptor, Treponema denticola, chymotrypsin-like protease, dentilisin

Abbreviations

CRT	Chemoradiotherapy
DSS	Disease-specific survival
DAMP	Danger-associated molecular pattern
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
HR	Hazard ratio
OPSCC	Oropharyngeal squamous cell carcinoma
PAMP	Pathogen-associated molecular pattern
SCC	Squamous cell carcinoma
Sx	Surgery
RT	Radiotherapy
TLR	Toll-like receptor
TMA	Tissue microarray
TNM	Tumor lymph node metastasis

Introduction

According to the latest WHO classification of head and neck tumors, the human papilloma virus (HPV)-associated oropharyngeal squamous cell carcinoma (OPSCC) and the HPV-unrelated OPSCC are distinct entities [4]. In developed countries, incidence of HPV-positive OPSCC is increasing [5-12], whereas incidence of HPV-negative OPSCC has remained stable or even declined [7,8,12]. Typically, patients carrying an HPV-positive tumor have better prognosis than patients with HPV-negative disease [5,6,11]. The differences now evident in the etiology and course of HPV-positive and -negative OPSCC will call for more detailed understanding of their cellular mechanisms and underlying causalities.

Toll-like receptors (TLRs) 1-10 participate in the initiation of innate immune cascades by recognizing pathogen-associated molecular patterns (PAMPs) of bacteria, viruses, fungi, and parasites, and recognizing endogenous danger-associated molecular patterns (DAMPs) in damaged or dying cells [13-15]. TLRs expressed in the immune cells contribute to chronic inflammation and an to attacking of tumor cells, whereas TLRs expressed during carcinogenesis by tumor cells may promote cancer-cell survival and chemoresistance [16,17]. The role of TLRs is, however, controversial, since TLR-activated immune cells may also show pro-tumorigenic effects [18,19]. The expression of TLRs may be prognostic in several cancers including colorectal-, salivary gland-, and non-small cell lung cancer, and squamous cell carcinoma of the tongue [20-24]. Recently, TLR5 and 7 in HPV-positive OPSCC have both shown the likelihood for providing prognostic value [1].

In oral and gastrointestinal tract cancers, the presence of several oral pathogens has been evident. Epidemiological studies have shown an association between the periodontal pathogen Porphyromonas gingivalis and increased risk of mortality from orodigestive cancer [25], and increased incidence of pancreatic cancer [26]. According to Narikiyo et al., [27] the oral pathogens Streptococcus mitis,

Streptococcus anginosus, and Treponema denticola (Td) are frequent in esophageal cancer tissue. Recently, Td has appeared also in oral- and gastrointestinal-tumor samples [28,29]. Furthermore, Fusobacterium nucleatum has been associated with colorectal cancer and shown to promote colorectal carcinogenesis [30]. Oral carcinogenesis, on the other hand, was in in vitro and in vivo studies promoted by F. nucleatum and P. gingivalis via their interaction with oral epithelial cells through TLR 2 [3]. In an in vitro and in vivo model of head and neck squamous cell carcinoma (HNSCC), the activation of TLR 2 promoted organoid growth, supporting its pro-tumorigenic role [2].

Earlier, we showed an association between Td key virulence factor, a chymotrypsin-like protease (Td-CTLP), and HPV-negative OPSCC [29]. We additionally observed that strong Td-CTLP expression associated with poor disease-specific survival. Here we aimed to investigate the presence of TLRs 2 and 4 in HPV-positive and HPV-negative OPSCC, and the relation of these to Td-CTLP expression, clinical parameters, and patient outcome in vivo in a series of 198 unselected consecutive OPSCC patients. Furthermore, we earlier proposed the use of HPV E6/E7 mRNA ISH detection method as a verifying test for p16-positive tumors [31], the concept which we here evaluate as a HPV classification method in parallel with the classification method proposed by Smeets et al. [32].

Materials and methods

Patients and clinicopathological data

This patient cohort, as previously described, [1] comprised 331 consecutive patients with oropharyngeal cancer diagnosed and treated at the Helsinki University Hospital (Finland) between 2000 and 2009. Included in the study were patients with ICD-10 codes C01, C02.4, C05.1, C05.2, C05.8, C05.9, C09.0,

C09.1, C09.8, C09.9, C10.0, C10.2, C10.3, C10.8, and C10.9 comprising squamous cell carcinomas (SCC) and subtypes of SCC. Patients treated with palliative treatment intent (44), with concurrent head and neck squamous cell carcinoma (HNSCC) (5), earlier treated HNSCC (11), histology other than SCC (18), or tumor-tissue unavailability (55) were considered ineligible for our investigation. The study series thus comprised 198 patients with treatment-naïve OPSCC treated with curative intention.

Clinicopathological data from patient records included patient age, sex; smoking and alcohol consumption; tumor histology, grade, UICC 7^{th} edition TNM stage [33]; primary treatment, i.e. surgery (Sx), radiotherapy (RT), and chemoradiotherapy (CRT); tumor recurrence, treatment of recurrent disease, and status at last follow-up [1]. Follow-up of all patients was at minimum three years or until death. Data on dates and causes of death came from Statistics Finland. This study received the approval of the Research Ethics Board of the Hospital District of Helsinki and Uusimaa and was granted institutional permission.

Among the 198 patients studied, 129 underwent primary surgery, and among these, 115 also were receiving adjuvant oncological treatment, either RT or CRT. Five patients received no postoperative oncological therapy because of their stage I-II disease and nine for patient-related reasons. Definitive CRT or RT was the treatment for 69 patients, and among these, 11 underwent salvage surgery in the primary treatment phase (primary site, one; neck only, 7; primary site and neck, 3). Tissue samples collected before RT/CRT involved all except the two patients who had only post-treatment samples available for immunohistochemistry.

Available from our earlier analysis were HPV DNA, HPV mRNA, $p16^{INK4a}$(p16) and Treponema denticola chymotrypsin-like protease (Td-CTLP) status [1,29,31]. Briefly, HPV DNA status was defined by Ventana Inform HPV in situ hybridization (ISH) assay using a high-risk HPV probe and an iVIEW

Blue detection kit in Benchmark XT series stainer machine (Ventana Medical Systems, Inc., Tuscon, AZ, USA). The assay has affinity to the high-risk HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 51, 56, 58, and 66. HPV DNA status was regarded as positive if any spot in the ISH assay was positive. HPV mRNA status was defined manually by the RNAscope®2.5 HD Reagent kit (Advanced Cell Diagnostics, Inc., Hayward, CA, USA) having affinity for E6/E7 HPV mRNA. HPV p16 and Td-CTLP immunohistochemical staining was performed on a tissue microarray (TMA) block slides prepared and stained as described previously [28,34]. We used monoclonal mouse anti-human $p16^{INK4a}$ (9517 CINtec Histology Kit, Roche Diagnostics, Basel, Switzerland) and polyclonal anti-CTLP IgG prepared according to the method of Grenier et al. [35] for immunostaining.

For evaluation purposes, three HPV-status classification methods were used. Data classification for Method A was as proposed by Smeets et al. [32] by dividing the samples into an HPV-positive group of 99 that included only p16-positive and HPV DNA-positive samples, and an HPV-negative group that included 99 either p16-positive but HPV DNA-negative samples, p16-negative and HPV DNA-negative samples, or p16-negative but HPV DNA-positive samples (Table 1). Similarly, a second classification (Method B) was as proposed by Randén-Brady et al., in which HPV DNA status was replaced by HPV mRNA status. The HPV-positive group included 106 p16-positive and HPV mRNA-positive samples; the HPV-negative group included 92 either p16-positive but HPV mRNA-negative samples, p16-negative and HPV mRNA-negative samples, or p16-negative but HPV mRNA -positive samples (Table 1). In third classification (Method C), the HPV-positive group included 109 HPV mRNA positive samples and HPV-negative group 89 HPV mRNA negative samples (Table 1).

Immunohistochemistry

We prepared TMA blocks, and stained slides by immunohistochemistry as described previously [34]. For expression of TLR 2 in the tumor TMA samples, the immunohistochemical staining was performed with polyclonal rabbit anti-human TLR 2 IgG (1:200, sc-10739, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and for TLR 4 with monoclonal mouse anti-human TLR 4 IgG (1:2000, sc-293072, Santa Cruz Biotechnology).

Immunoscore

The decoded TMA slides immunostained with TLR 2 and 4 antibody separately underwent scoring by two researchers (J.H. and A.K.K.). In case of discordance, the slides were reassessed to achieve consensus. TLR scoring was based on intensity of positivity in tumor tissue: none (0), mild (1), moderate (2), or strong (3). The highest immunoscore value for each TLR was chosen for analysis when several tumor spots were available for selection. Positivity in TLR 2 and 4 staining was localized in the cytoplasm of the carcinoma cells. In addition to carcinoma cells, TLR2 was detected in tumor infiltrating lymphocytes and TLR4 especially in plasma cells, but these were not scored, and hence are not included in analysis. (Figure 1)

Statistical analysis

We used SPSS version 20.0 (IBM SPSS Statistics, IBM Corporation, New York, NY, USA) for statistical data analysis. Differences between categorical variables we evaluated using the Chi-square test with asymptotic or exact P-value, whichever appropriate. The Kaplan-Meier (KM) estimator served in calculation of 5-year disease-specific survival (DSS) rates. In evaluation of statistically significant

between-group differences in DSS, we used the log-rank test. We defined follow-up time in the DSS as the period between the last treatment day and the last day of follow-up or date of death from the disease. To minimize bias in the follow-up, the maximum follow-up period for the analysis was five years. The Cox proportional hazard model we utilized for multivariable survival analysis. This analysis included clinically relevant variables with a P-value less than 0.1. The proportional hazard assumptions were tested with KM curves. A two-sided P-value less than 0.05 was considered statistically significant.

Results

TLR 2 and 4 association with patient and tumor characteristics in OPSCC

TLR 2 and TLR 4 expression appeared in the cytoplasm of OPSCC cells. Among the 198 samples, TLR 2 was immunoexpressed as follows: 7 (4%) had none, 63 (32%) were mild, 98 (49%) moderate, and 30 (15%) strong. As for TLR 4 immunoexpression: 22 (11%) had none, 71 (36%) were mild, 69 (35%) moderate, and 36 (18%) strong.

TLR 2 showed a significant association with patient alcohol consumption (Table 2). Mild and moderate TLR 2 immunoexpression prevailed among all alcohol-consumption groups (none, former, and current excess alcohol consumption habit). Strong immunoexpression of TLR 2 was observable only among patients with none or former excess alcohol consumption. Among tumors with strong TLR 2 immunoexpression, 89% were those of patients with no excess alcohol consumption.

TLR 4 was associated with patient's alcohol consumption and grade of tumor differentiation (Table 3). As with TLR 2, mild and moderate TLR 4 immunoexpression prevailed among all alcohol consumption groups, but strong TLR 4 immunoexpression appeared among all alcohol consumption groups. Grade 1 tumors had none to moderate TLR 4 immunoexpression (score 0-2), with mild expression dominating. Strong TLR 4 immunoexpression existed only in tumors of grade 2 (23%) and 3 (77%) (Table 3).

TLR 2 and 4 association with HPV status

Immunoexpression of both TLR 2 and TLR 4 showed significant association with HPV status when method A and B were used for classification (Table 4). A significant association was also detectable when method C served as a classification method (TLR2, P=0.009; TLR4, P=0.005). Strong expression was associated with HPV positivity and mild expression with HPV negativity (Table 4).

TLR 2 and 4 association with Td-CTLP

No association existed between TLR 2 or 4 and Td-CTLP immunoexpression (Table 4).

TLR 2 and 4 association with survival

In survival analysis of all OPSCC patients, and in comparison of sample subgroups with TLR 2 staining of none to mild (0-1) and moderate to strong (2-3), no statistically significant difference emerged in the 5-year DSS (P=0.074). Among patients with HPV-negative OPSCC according to method A, the 5-year DSS was 77% among patients with TLR 2 score 0-1 and 58% among those of score moderate to strong

TLR 2 (P=0.032). When method B was used for HPV status classification, the association of TLR 2 with DSS in the HPV-negative group was non-significant (P=0.057) (Figure 2). When HPV status was classified according to method C, the association between TLR 2 score and DSS among HPV-negative OPSCC was again significant (P=0.046). Among patients with HPV-positive OPSCC, TLR 2 had no association with DSS (P=0.092 (A); P=0.054 (B); P=0.052 (C)). TLR 4 had no associations with DSS among all OPSCC or in any of the subgroups based on classification methods A, B or C.

In multivariable analysis of all 198 patients, sex, TLR 2, and HPV (method A) were independent prognostic factors having respective hazard ratios of 2.5, 2.6 and 3.0 (P=0.028; P=0.010; P=0.007) (Table 5).

Discussion

We show here that mild TLR 2 immunoexpression associated with negative HPV status in OPSCC, and further, that patients with strong TLR 2 immunoexpression in the HPV-negative subgroup had significantly poorer 5-year DSS (58%) than did patients with mild TLR 2 expression (77%). Strong TLR 2 immunoexpression remained as an independent factor and was related to increased disease mortality in the multivariable setting. Patients with strong TLR 2 expression had a trend towards a poorer DSS also in the HPV-positive OPSCC subgroup, and among all OPSCC patients, but these differences were not statistically significant. Our findings are in line with findings of Farnebo et al. indicating that TLR 2-receptor activation mediates pro-tumorigenic effects in vivo in HNSCC xenograft tumors [2]. In their study, inhibition of TLR 2-receptor also inhibited tumor growth in tumor xenografts in vivo and in HNSCC cell lines in vitro [2]. Similarly, according to findings of Binder Gallimidi et al., TLR 2 inhibition

resulted in an anti-tumorigenic effect on cell proliferation and inhibited the oral pathogen-induced Il-6 expression in oral SCC cell lines [3]. Here we may hypothesize that strong TLR2 expression among HPV-negative OPSCC is related mainly to carcinogenesis, whereas among HPV-positive OPSCC there may be a component of TLR2 expression related to virus infection.

The significance of the association of TLR 2 immunoexpression with DSS in HPV-negative OPSCC patients was dependent on the method used for HPV status classification. According to classification method A proposed by Smeets et al. the number of HPV-positive OPSCC samples was smaller than when method B proposed by Randén-Brady et al. was used for classification [31,32]. This is explained by the higher sensitivity of the HPV mRNA method compared with that of the HPV DNA method [31]. The number of HPV-positive OPSCC samples was even greater when HPV mRNA alone in method C was used for classification. However, the differences in significance were relatively small (P=0.032 (A); P=0.054 (B); P=0.046 (C)) and reflect our limited number of OPSCC samples in this study, only 198. We may speculate that with a higher number of OPSCC samples, the association of TLR 2 expression with DSS would be statistically significant in HPV-negative OPSCC independent of classification method. Hence, our results comprise no contraindication to our earlier proposal to use HPV mRNA detection method to verify p16-positive tumors [31].

Of the OPSCC samples TLR 2 was expressed in 96% and TLR 4 in 89%, but their expression showed no association with Td-CTLP immunoexpression, although both TLR 2 and 4 recognize bacterial components [36]. Furthermore, Td is able to trigger TLR 2 and 4 in murine macrophages [37]. Interestingly, strong expression of TLR 2 and 4 was associated with HPV positivity. Since HPV infection has an association with periodontitis [38,39], a dysbiosis contributed to several opportunistic oral pathogens, the strong TLR 2 and 4 may result from activation by bacteria other than those studied here. The role of oral pathogens in triggering TLR 2 receptor gained earlier support when oral SCC cell lines

derived from tongue cancer tissue were exposed to the oral pathogens Porphyromonas gingivalis or Fusobacterium nucleatum, or both of them [3]. TLR 2 and 4 positivity is usually detectable on cell membranes [40] whereas here the immunopositivity was cytoplasmic. This may reflect the more diverse role of TLRs in carcinogenesis when compared with their role in microbial activation [41].

TLR 2 immunoexpression was associated with alcohol consumption, and 89% of OPSCC samples with strong TLR 2 expression occurred among those patients with no excess alcohol consumption. This is consistent with earlier results, in which HPV-positive OPSCC is more likely to occur among patients using less alcohol [5], and strong TLR 2 expression was in our series more common among HPV-positive OPSCC.

We also showed that TLR 4 was associated with alcohol consumption and with tumor grade of differentiation. Over 50% of the tumors expressing strong TLR 4 appeared among non-alcohol users and were either grade 2 or 3. As strong TLR 4 expression was also associated with HPV positivity, these findings are in line with the current clinicopathological characteristics reported for HPV-positive OPSCC, indicating that patients with HPV-positive tumors are less often heavy alcohol users [42,43]. The association between TLR 2 and 4 and alcohol consumption itself may be related to alcohol mediated inflammatory responses, and thus will be the subject of another study.

In our recent study with the same patient series, we observed Td-CTLP in 81% of the OPSCC samples [29], and in the HPV-negative subgroup of OPSCC samples showed an association of strong Td-CTLP immunoexpression with strong TLR 5 expression and with mild TLR 7 expression [1,29]. Further, strong Td-CTLP immunoexpression among patients with HPV-negative OPSCC was associated with a poor DSS [29]. Our current results show an association between strong TLR 2 immunoexpression and poor

DSS among the same HPV-negative subgroup of OPSCC and support the role of TLR 2 receptor as a possible target for development of therapeutics as proposed earlier [2].

Based on the present results it is impossible to conclude that involvement of Td would trigger the TLR 2 or TLR 4 receptors. However, earlier findings have demonstrated the triggering of TLR 2 receptor by the oral pathogens P. gingivalis and F. nucleatum [3]; this may suggest that Td and other oral pathogens also play a role in oral carcinogenesis. In periodontitis, the periodontopathogens act in combinations of several bacteria, leading the dysbiosis in the gingival microbiota [26]. Interestingly, here we observed that strong Td-CTLP expression was more common in OPSCC samples which showed no expression to mild TLR 4 expression, although that association was statistically non-significant (P=0.080). This is in line with our earlier finding of strong Td-CTLP expression as being associated with HPV negativity [29]. Nonetheless, the involvement of Td and other oral pathogens in carcinogenesis of OPSCC, and also in host immunological responses, remains an open possibility.

Conclusions

The present results show an association between strong TLR 2 immunoexpression and poor DSS among the HPV-negative subgroup of OPSCC patients and thus support the role of TLR 2 receptor as a possible target for the development of therapeutics for this disease as proposed earlier [2]. This view is further strengthened here, because strong TLR 2 immunoexpression remained in multivariable analysis an independent factor that elevated disease mortality.

The involvement of Td and other oral pathogens in carcinogenesis of OPSCC, and also in host immunological responses, calls for further study.

Ethics approval

The study received institutional permission and the approval of The Research Ethics Board of the Hospital District of Helsinki and Uusimaa, Finland.

Funding

This work was supported by the Minerva Foundation Institute for Medical Research, grant number 701337.

Role of the Funding Source

Minerva Foundation provided partial funding for the research work based on grant application and a research plan.

Acknowledgements

We thank Päivi Peltokangas and Pia Saarinen for technical assistance.

References

[1] Jouhi L, Mohamed H, Makitie A, Remes SM, Haglund C, Atula T, et al. Toll-like receptor 5 and 7 expression may impact prognosis of HPV-positive oropharyngeal squamous cell carcinoma patients. Cancer Immunol.Immunother. 2017.

[2] Farnebo L, Shahangian A, Lee Y, Shin JH, Scheeren FA, Sunwoo JB. Targeting Toll-like receptor 2 inhibits growth of head and neck squamous cell carcinoma. Oncotarget 2015;6:9897-907.

[3] Binder Gallimidi A, Fischman S, Revach B, Bulvik R, Maliutina A, Rubinstein AM, et al. Periodontal pathogens Porphyromonas gingivalis and Fusobacterium nucleatum promote tumor progression in an oral-specific chemical carcinogenesis model. Oncotarget 2015;6:22613-23.

[4] El-Naggar AK, Chan J, Takata T, Grandis J, Slootweg P. The 4th Edition of the Head and Neck WHO Blue Book: Editor's Perspectives. Hum.Pathol. 2017.

[5] Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J.Natl.Cancer Inst. 2000;92:709-20.

[6] Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N.Engl.J.Med. 2010;363:24-35.

[7] Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J.Clin.Oncol. 2011;29:4294-301.

[8] Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? Int.J.Cancer 2009;125:362-6.

[9] Nichols AC, Palma DA, Dhaliwal SS, Tan S, Theuer J, Chow W, et al. The epidemic of human papillomavirus and oropharyngeal cancer in a Canadian population. Curr.Oncol. 2013;20:212-9.

[10] Rietbergen MM, Leemans CR, Bloemena E, Heideman DA, Braakhuis BJ, Hesselink AT, et al. Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in the Netherlands as assessed by a validated test algorithm. Int.J.Cancer 2013;132:1565-71.

[11] Reuschenbach M, Tinhofer I, Wittekindt C, Wagner S, Klussmann JP. A systematic review of the HPV-attributable fraction of oropharyngeal squamous cell carcinomas in Germany. Cancer.Med. 2019;8:1908-18.

[12] Wittekindt C, Wagner S, Bushnak A, Prigge ES, von Knebel Doeberitz M, Wurdemann N, et al. Increasing Incidence rates of Oropharyngeal Squamous Cell Carcinoma in Germany and Significance of Disease Burden Attributed to Human Papillomavirus. Cancer.Prev.Res.(Phila) 2019;12:375-82.

[13] Janeway CA,Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb.Symp.Quant.Biol. 1989;54 Pt 1:1-13.

[14] Matzinger P. The danger model: a renewed sense of self. Science 2002;296:301-5.

[15] Kawai T, Akira S. Pathogen recognition with Toll-like receptors. Curr.Opin.Immunol. 2005;17:338-44.

[16] Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Arch.Pharm.Res. 2012;35:1297-316.

[17] Ioannou S, Voulgarelis M. Toll-like receptors, tissue injury, and tumourigenesis. Mediators Inflamm. 2010;2010:10.1155/2010/581837. Epub 2010 Sep 14.

[18] Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. J.Clin.Invest. 2006;116:485-94.

[19] Hussaini HM, Parachuru VPB, Seymour GJ, Rich AM. Forkhead box-P3(+) regulatory T cells and toll-like receptor 2 co-expression in oral squamous cell carcinoma. Acta Histochem. 2017;119:205-10.

[20] Grimm M, Kim M, Rosenwald A, Heemann U, Germer CT, Waaga-Gasser AM, et al. Toll-like receptor (TLR) 7 and TLR8 expression on CD133+ cells in colorectal cancer points to a specific role for inflammation-induced TLRs in tumourigenesis and tumour progression. Eur.J.Cancer 2010;46:2849-57.

[21] Korvala J, Harjula T, Siirila K, Almangush A, Aro K, Makitie AA, et al. Toll-like receptor 9 expression in mucoepidermoid salivary gland carcinoma may associate with good prognosis. J.Oral Pathol.Med. 2014;43:530-7.

[22] Kauppila JH, Mattila AE, Karttunen TJ, Salo T. Toll-like receptor 5 (TLR5) expression is a novel predictive marker for recurrence and survival in squamous cell carcinoma of the tongue. Br.J.Cancer 2013;108:638-43.

[23] Makinen LK, Atula T, Hayry V, Jouhi L, Datta N, Lehtonen S, et al. Predictive role of Toll-like receptors 2, 4, and 9 in oral tongue squamous cell carcinoma. Oral Oncol. 2015;51:96-102.

[24] Zhou H, Chen JH, Hu J, Luo YZ, Li F, Xiao L, et al. High expression of Toll-like receptor 5 correlates with better prognosis in non-small-cell lung cancer: an anti-tumor effect of TLR5 signaling in non-small cell lung cancer. J.Cancer Res.Clin.Oncol. 2014;140:633-43.

[25] Ahn J, Segers S, Hayes RB. Periodontal disease, Porphyromonas gingivalis serum antibody levels and orodigestive cancer mortality. Carcinogenesis 2012;33:1055-8.

[26] Michaud DS, Izard J, Wilhelm-Benartzi CS, You DH, Grote VA, Tjonneland A, et al. Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. Gut 2013;62:1764-70.

[27] Narikiyo M, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, et al. Frequent and preferential infection of Treponema denticola, Streptococcus mitis, and Streptococcus anginosus in esophageal cancers. Cancer.Sci. 2004;95:569-74.

[28] Nieminen MT, Listyarifah D, Hagstrom J, Haglund C, Grenier D, Nordstrom D, et al. Treponema denticola chymotrypsin-like proteinase may contribute to orodigestive carcinogenesis through immunomodulation. Br.J.Cancer 2018;118:428-34.

[29] Kylma AK, Jouhi L, Listyarifah D, Mohamed H, Makitie A, Remes SM, et al. Treponema denticola chymotrypsin-like protease as associated with HPV-negative oropharyngeal squamous cell carcinoma. Br.J.Cancer 2018;119:89-95.

[30] Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell host & microbe 2013;14:195-206.

[31] Randen-Brady R, Carpen T, Jouhi L, Syrjanen S, Haglund C, Tarkkanen J, et al. In situ hybridization for high-risk HPV E6/E7 mRNA is a superior method for detecting transcriptionally active HPV in oropharyngeal cancer. Hum.Pathol. 2019;90:97-105.

[32] Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int.J.Cancer 2007;121:2465-72.

[33] Anonymous In:Sobin LH, Gospodarowicz MK, Wittekind C, editors. TNM classification of malignant tumours, Hoboken, New Jersey, USA:Wiley-Blackwell;2010,.

[34] Makinen LK, Hayry V, Atula T, Haglund C, Keski-Santti H, Leivo I, et al. Prognostic significance of matrix metalloproteinase-2, -8, -9, and -13 in oral tongue cancer. J.Oral Pathol.Med. 2012;41:394-9.

[35] Grenier D, Uitto VJ, McBride BC. Cellular location of a Treponema denticola chymotrypsinlike protease and importance of the protease in migration through the basement membrane. Infect.Immun. 1990;58:347-51.

[36] Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443-51.

[37] Nussbaum G, Ben-Adi S, Genzler T, Sela M, Rosen G. Involvement of Toll-like receptors 2 and 4 in the innate immune response to Treponema denticola and its outer sheath components. Infect.Immun. 2009;77:3939-47.

[38] Ortiz AP, Gonzalez D, Vivaldi-Oliver J, Castaneda M, Rivera V, Diaz E, et al. Periodontitis and oral human papillomavirus infection among Hispanic adults. Papillomavirus Res. 2018;5:128-33.

[39] Tezal M, Scannapieco FA, Wactawski-Wende J, Hyland A, Marshall JR, Rigual NR, et al. Local inflammation and human papillomavirus status of head and neck cancers. Arch.Otolaryngol.Head.Neck.Surg. 2012;138:669-75.

[40] Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu.Rev.Immunol. 2003;21:335-76.

[41] Jouhi L, Renkonen S, Atula T, Makitie A, Haglund C, Hagstrom J. Different Toll-Like Receptor Expression Patterns in Progression toward Cancer. Front.Immunol. 2014;5:638.

[42] Nauta IH, Rietbergen MM, van Bokhoven AAJD, Bloemena E, Lissenberg-Witte BI, Heideman DAM, et al. Evaluation of the eighth TNM classification on p16-positive oropharyngeal squamous cell carcinomas in the Netherlands and the importance of additional HPV DNA testing. Ann.Oncol. 2018;29:1273-9.

[43] Gillison ML. Human Papillomavirus and Oropharyngeal Cancer Stage. J.Clin.Oncol. 2016;34:1833-5.

Table 1 Classification of oropharyngeal squamous cell carcinoma (OPSCC) according to methods A, B and C based on results of detection methods $p16^{INK4a}$, HPV DNA and HPV mRNA.

	Detection method			All patients (N =198)
HPV-status	**p16INK4a**	**HPV DNA**	**HPV mRNA**	**n**
Method A (Smeets *et al*., 2007)				
HPV-positive	positive	positive	-	99
HPV-negative	positive	negative	-	99
	negative	negative	-	
	negative	positive	-	
Method B (Randén-Brady *et al*., 2019)				
HPV-positive	positive	-	positive	106
HPV-negative	positive	-	negative	92
	negative	-	negative	
	negative	-	negative	
Method C				
HPV-positive	-	-	positive	109
HPV-negative	-	-	negative	89

Table 2 Toll-like receptor 2 (TLR 2) immunoexpression in relation to patient and tumor characteristics; patient sex, smoking and alcohol consumption, tumor site of origin, grade of differentiation, T class (primary tumor size), N class (presence of regional lymph node metastasis), and tumor stage.

		TLR 2 score								
	Total	None (0)		Mild (1)		Moderate (2)		Strong (3)		*P* value
		n	*%*	n	*%*	n	*%*	n	*%*	
Sex										0.470
Male	146	5	*3.4*	48	*32.9*	73	*50.0*	20	*13.7*	
Female	52	2	*3.8*	15	*28.8*	25	*48.1*	10	*19.2*	
Smoking										0.575
None	26	1	*3.8*	6	*23.1*	15	*57.7*	4	*15.4*	
Finished	48	1	*2.1*	15	*31.3*	23	*47.9*	9	*18.8*	
Current	94	3	*3.2*	30	*31.9*	47	*50.0*	14	*14.9*	
Excess alcohol consumption										**0.001**
None	60	1	*1.7*	16	*26.7*	26	*43.3*	17	*28.3*	
Finished	24	0	*0.0*	8	*33.3*	14	*58.3*	2	*8.3*	
Current	37	5	*13.5*	11	*29.7*	21	*56.8*	0	*0.0*	
Tumor site										0.579
Anterior wall	59	3	*5.1*	16	*27.1*	31	*52.5*	9	*15.3*	
Lateral wall	115	3	*2.6*	37	*32.2*	57	*49.6*	18	*15.7*	
Posterior wall	3	1	*33.3*	1	*33.3*	1	*33.3*	0	*0.0*	
Superior wall	21	0	*0.0*	9	*42.9*	9	*42.9*	3	*14.3*	
Tumor grade										0.080
Grade 1	18	1	*5.6*	4	*22.2*	11	*61.1*	2	*11.1*	
Grade 2	77	4	*5.2*	28	*36.4*	40	*51.9*	5	*6.5*	
Grade 3	103	2	*1.9*	31	*30.1*	47	*45.6*	23	*22.3*	
T class										1.000
T1-2	112	5	*4.5*	35	*31.3*	54	*48.2*	18	*16.1*	
T1-3	86	2	*2.3*	28	*32.6*	44	*51.2*	12	*14.0*	
N class										0.471
N0	38	1	*2.6*	15	*39.5*	17	*44.7*	5	*13.2*	
N+	160	6	*3.8*	48	*30.0*	81	*50.6*	25	*15.6*	
Tumor stage										0.284
I-II	29	1	*3.4*	12	*41.4*	13	*44.8*	3	*10.3*	
III-IV	169	6	*3.6*	51	*30.2*	85	*50.3*	27	*16.0*	

Table 3 Toll-like receptor 4 (TLR 4) immunoexpression in relation to patient and tumor characteristics; patient sex, smoking and alcohol consumption, tumor site of origin, grade of differentiation, T class (primary tumor size), N class (presence of regional lymph node metastasis), and tumor stage.

		TLR 4 score								
	Total	None (0)		Mild (1)		Moderate (2)		Strong (3)		*P* value
		n	*%*	n	*%*	n	*%*	n	*%*	
Sex										0.717
Male	146	15	*10.3*	58	*39.7*	49	*33.6*	24	*16.4*	
Female	52	7	*13.5*	13	*25.0*	20	*38.5*	12	*23.1*	
Smoking										0.119
None	26	1	*3.8*	8	*30.8*	14	*53.8*	3	*11.5*	
Finished	48	4	*8.3*	17	*35.4*	16	*33.3*	11	*22.9*	
Current	94	15	*16.0*	34	*36.2*	30	*31.9*	15	*16.0*	
Excess alcohol consumption										**0.022**
None	60	8	*13.3*	17	*28.3*	25	*41.7*	10	*16.7*	
Finished	24	2	*8.3*	10	*41.7*	9	*37.5*	3	*12.5*	
Current	37	9	*24.3*	17	*45.9*	7	*18.9*	4	*10.8*	
Tumor site										0.928
Anterior wall	59	9	*15.3*	22	*37.3*	26	*44.1*	2	*3.4*	
Lateral wall	115	11	*9.6*	39	*33.9*	33	*28.7*	32	*27.8*	
Posterior wall	3	1	*33.3*	1	*33.3*	1	*33.3*	0	*0.0*	
Superior wall	21	3	*14.3*	9	*42.9*	8	*38.1*	1	*4.8*	
Tumor grade										**0.001**
Grade 1	18	4	*22.2*	10	*55.6*	4	*22.2*	0	*0.0*	
Grade 2	77	6	*7.8*	37	*48.1*	26	*33.8*	8	*10.4*	
Grade 3	103	14	*13.6*	24	*23.3*	38	*36.9*	27	*26.2*	
T class										0.695
T1-2	112	15	*13.4*	38	*33.9*	36	*32.1*	23	*20.5*	
T1-3	86	9	*10.5*	33	*38.4*	32	*37.2*	12	*14.0*	
N class										0.338
N0	38	5	*13.2*	16	*42.1*	12	*31.6*	5	*13.2*	
N+	160	19	*11.9*	55	*34.4*	56	*35.0*	30	*18.8*	
Tumor stage										0.092
I-II	29	4	*13.8*	15	*51.7*	7	*24.1*	3	*10.3*	
III-IV	169	20	*11.8*	56	*33.1*	61	*36.1*	32	*18.9*	

Table 4 Toll-like receptor 2 (TLR 2) and TLR 4 immunoexpression in relation to oropharyngeal squamous cell carcinoma (OPSCC) human papillomavirus (HPV) status classified using p16, HPV DNA, and HPV mRNA results, and Treponema denticola chymotrypsin-like protease (Td-CTLP) immunoexpression.

		TLR2 score									TLR4 score								
	Total	**None (0)**		**Mild (1)**		**Moderate (2)**		**Strong (3)**		***P* value**	**None (0)**		**Mild (1)**		**Moderate (2)**		**Strong (3)**		***P* value**
		n	*%*	n	*%*	n	*%*	n	*%*		n	*%*	n	*%*	n	*%*	n	*%*	
HPV status[A]										**0.001**									***P* < 0.001**
HPV-negative	99	5	*5.1*	42	*42.4*	41	*41.4*	11	*11.1*		20	*20.2*	46	*46.5*	24	*24.2*	9	*9.1*	
HPV-positive	99	2	*2.0*	21	*21.2*	57	*57.6*	19	*19.2*		4	*4.0*	25	*25.3*	44	*44.4*	26	*26.3*	
HPV status[B]										**0.007**									***P* < 0.001**
HPV-negative	92	3	*3.3*	39	*42.4*	41	*44.6*	9	*9.8*		19	*20.7*	41	*44.6*	23	*25.0*	9	*9.8*	
HPV-positive	106	4	*3.8*	24	*22.6*	57	*53.8*	21	*19.8*		5	*4.7*	30	*28.3*	45	*42.5*	26	*24.5*	
***Td*-CTLP expression**										0.634									0.080
0 - 1	98	5	*5.1*	28	*28.6*	47	*48.0*	18	*18.4*		9	*9.2*	32	*32.7*	37	*37.8*	20	*20.4*	
2 - 3	99	2	*2.0*	34	*34.3*	51	*51.5*	12	*12.1*		15	*15.2*	38	*38.4*	31	*31.3*	15	*15.2*	

[A] = HPV classification method A

[B] = HPV classification method B

Table 5 Multivariable Cox regression analysis for Disease specific survival (DSS) in a series of all 198 oropharyngeal squamous cell carcinoma (OPSCC) patients.

	Multivariabe analysis All patients (N =198)		
	HR	**95% CI**	***P***
Sex			
Female vs male	2.5	1.1 -5.6	**0.028**
Smoking			
Currently vs earlier/never	1.4	0.7 - 3.1	0.362
T class			
T3-4 vs T1-2	1.3	0.7 - 2.4	0.35
N class			
N+ vs N0	1.8	0.7 - 4.5	0.203
TLR 2			
2-3 vs 0-1	2.6	1.3 - 5.3	**0.010**
HPV status[A]			
HPV-negative vs HPV-positive	3.0	1.4 - 6.5	**0.007**

[A] = HPV classificatio method A

485 **Figure 1.** Immunohistochemical staining of oropharyngeal squamous cell carcinoma (OPSCC) samples
486 with Toll-like receptor (TLR) 2 and TLR4 antibody. OPSCC with none **A**, mild **B**, moderate **C**, and
487 strong **D** expression of TLR2, and OPSCC with none **E**, mild **F**, moderate **G**, and strong **H** expression
488 of TLR4. Arrowheads point to the tumor tissue, and arrows to the infiltrating lymphocytes and plasma
489 cells. Magnification ×200.

490

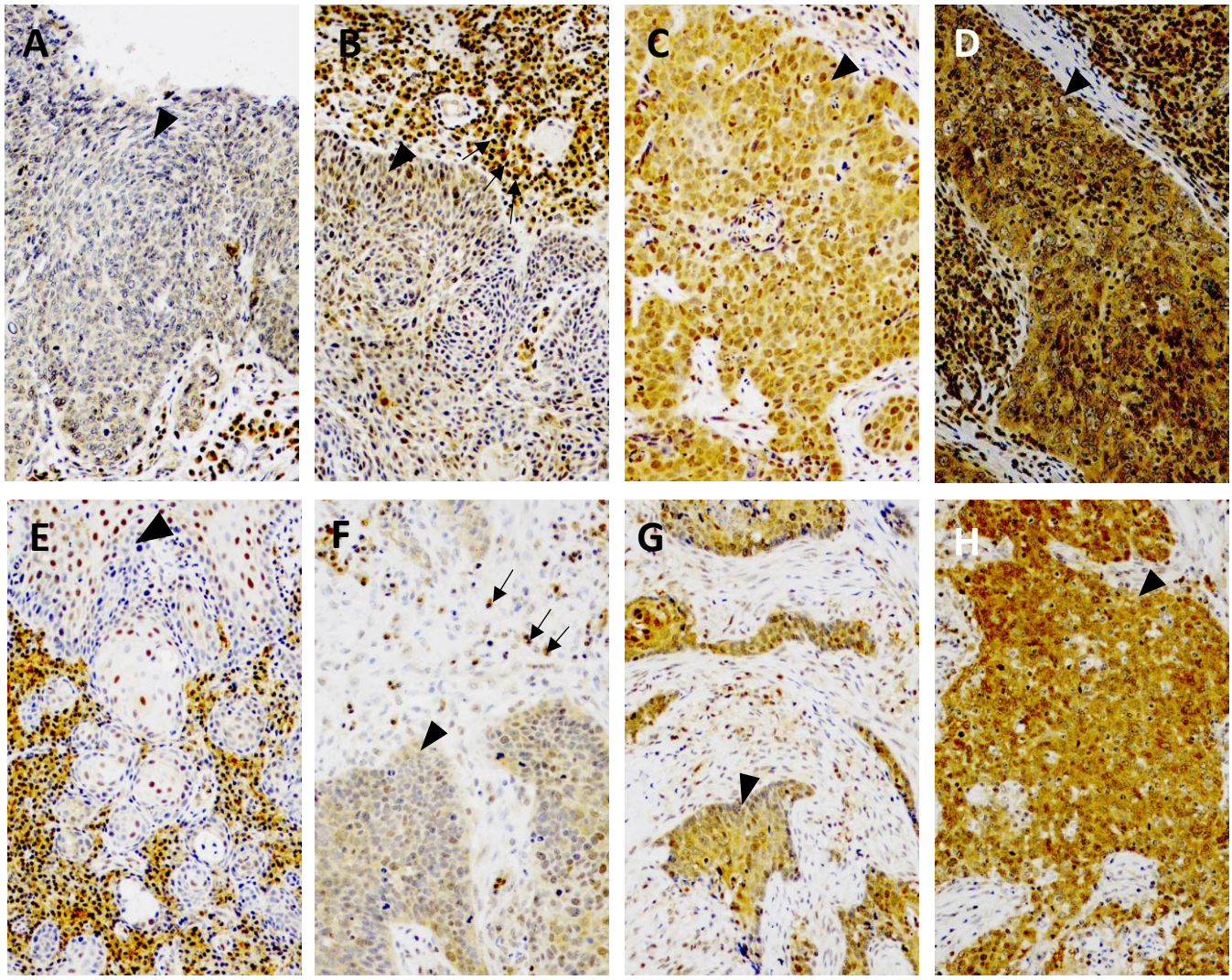
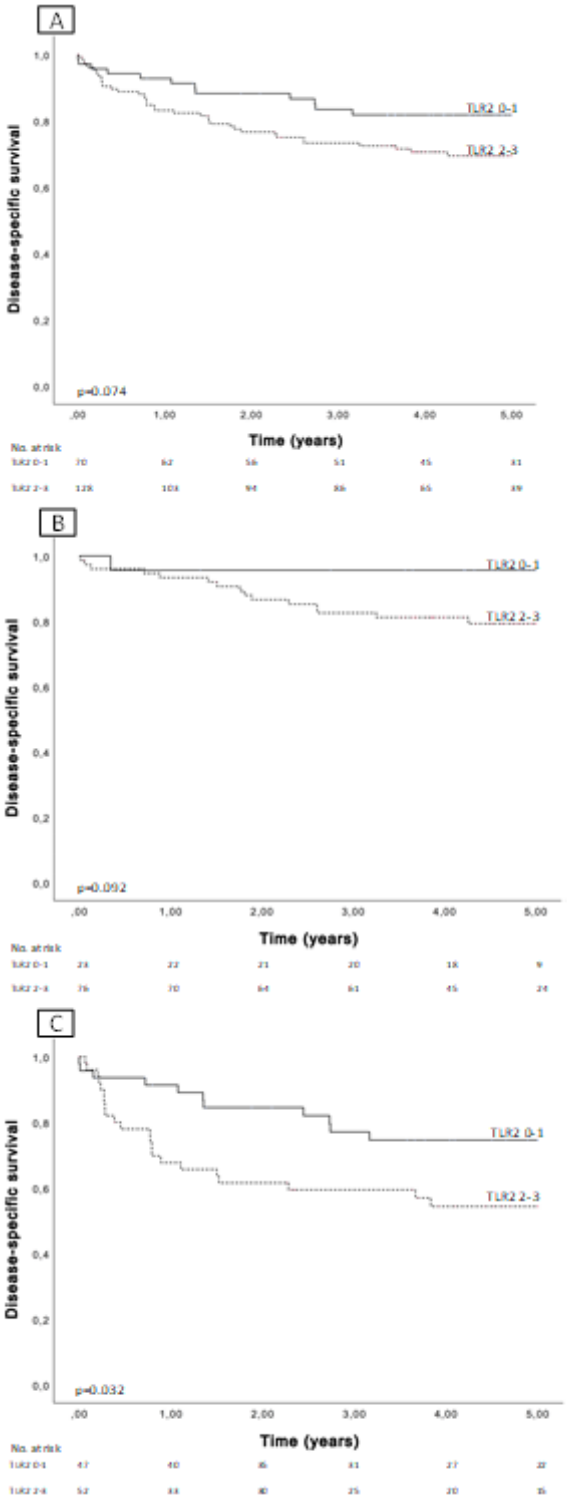


Figure 2. The 5-year disease-specific survival (DSS) rates calculated by Kaplan-Meier (KM) estimation for the entire sample (A) and for the human papillomavirus (HPV) -positive (B) and the HPV-negative (C) subgroups classified by p16- and HPV DNA results. Samples grouped according to toll-like receptor 2 (TLR 2) immunoexpression into the categories none to mild (score = 0 – 1) and moderate to strong (score 2 - 3).


A
Disease-specific survival
1,0
0,8
0,6
0,4
0,2
0,0
TLR2 0-1
TLR2 2-3
p=0.074
,00
1,00
2,00
3,00
4,00
5,00
Time (years)
No. at risk
B
Disease-specific survival
TLR2 0-1
TLR2 2-3
p=0.092
Time (years)
No. at risk
C
Disease-specific survival
TLR2 0-1
TLR2 2-3
p=0.032
Time (years)
No. at risk

496