ANTICANCER RESEARCH *42*: 5415-5430 (2022)
doi:10.21873/anticanres.16046

Prognostic Role of *Porphyromonas gingivalis* Gingipain Rgp and Matrix Metalloproteinase 9 in Oropharyngeal Squamous Cell Carcinoma

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Abstract. *Background/Aim: The oral bacteria involved in the development of periodontitis alter the tissue conditions and modify immune responses in a way that may also influence tumor development. We investigated the prevalence of R gingipain (Rgp), a key virulence factor of the oral pathobiont Porphyromonas gingivalis, and the tissue-destructive enzymes matrix metalloproteinase 8 (MMP-8) and 9 (MMP-9) in 202 unselected consecutive oropharyngeal squamous cell carcinoma (OPSCC) samples. We further investigated the relationships between these factors and human papillomavirus (HPV) status, Treponema denticola chymotrypsin-like proteinase (Td-CTLP) immunoexpression, clinical parameters, and patient outcome. Patients and Methods: Clinicopathological data were derived from university hospital records. Rgp, MMP-8, and MMP-9 immunoexpression was evaluated by immunohistochemistry; the immunohistochemistry of Td-CTLP and HPV has been described earlier for this patient series. Cox regression analysis including death by causes other than OPSCC as a competing risk served to assess sub distribution hazard ratios. Results: In multivariable survival analysis, positive tumoral MMP-9 immunoexpression predicted poor prognosis among all patients [sub distribution hazard ratio (SHR)=2.4; confidence interval (CI)=1.2-4.4, p=0.008], and especially among those with HPV-negative OPSCC (SHR=3.5; CI=1.7-7.3, p=0.001). Positive immunoexpression of Rgp in inflammatory cells was associated with favorable outcome among all patients (SHR=0.5, CI=0.2-0.9, p=0.021) and among those with HPV-negative disease (SHR=0.4, CI=0.2-0.9, p=0.022). Conclusion: Our results suggest that tumoral MMP-9 may be related to poor outcome in OPSCC, especially in HPV-negative disease, while Rgp immunoexpression in inflammatory cells is associated here with better disease-specific survival (DSS).*

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Key Words: Oropharyngeal squamous cell carcinoma, OPSCC, human papillomavirus, *Porphyromonas gingivalis*, gingipain, Rgp, matrix metalloproteinase, survival analysis.

Microbial infection is estimated to play a role in nearly 20% of all malignancies (1). Since the acknowledgement of Helicobacter pylori as a causative agent of gastric cancer in 1994 (2), it has become more and more apparent that it is important to understand the role and the long-term effects of bacteria in order to develop better tools for cancer prevention.

The presence of several oral pathogens has been evident in oral and gastrointestinal tract cancers. The dysbiotic periodontal pathobiont *Porphyromonas gingivalis* (Pg) has occurred in abundance in oral squamous cell carcinoma (OSCC) (3), and further, Pg has been associated with an increased risk of mortality from orodigestive cancer (4), and with an increased incidence of pancreatic cancer (5). In esophageal cancer tissue, the oral pathogens *Streptococcus mitis*, *Streptococcus anginosus*, and *Treponema denticola* (Td) are frequent (6), and in our earlier studies, Td has appeared in oropharyngeal, oral- and gastrointestinal tumor samples (7, 8). In colorectal cancer, *Fusobacterium nucleatum* (Fn) has been present and evidence shows that it promotes colorectal carcinogenesis (9). Oral carcinogenesis has been promoted by Fn and Pg in *in vitro* and *in vivo* studies *via* their interaction with oral epithelial cells through toll-like receptors (TLR) (10). Pg is an invasive opportunistic pathobiont belonging to a red complex group of oral pathogens identifiable in severe forms of periodontitis (11). In addition to its association with gastrointestinal cancers, Pg has been associated with various systemic diseases including rheumatoid arthritis (12) and Alzheimer's disease (13). The tumorigenic properties of Pg include induction of inflammation (14), activation of cell proliferation (15), inhibition of apoptosis (16), enhancement of cell invasion (17), epithelial mesenchymal transition (EMT) (18) and immune suppression (19). One of its key virulence factors, arginine-specific cysteine proteinase R gingipain (Rgp), has several functions: it can degrade host structural elements and thus contribute to Pg penetration into epithelium and to induction of cell apoptosis (20), as well as participate in altering host defenses by manipulating inflammatory responses (21). Pg activates host responses, leading to increased pro-matrix metalloproteinase 9 (proMMP-9) and proMMP-8 expression and activation (17, 22, 23), while gingipains activate proMMP-9 and proMMP-8 to their active form, thus enhancing extracellular matrix destruction and cell penetration (17, 24).

Matrix metalloproteinases (MMPs) derived from host cells are capable of degrading almost all components of extracellular matrix and basement membranes. MMPs are essential in many physiological processes requiring tissue remodeling such as angiogenesis, bone development, wound healing and uterine and mammary involution (25). They also play a critical role in inflammatory and immunological processes; upregulation of MMPs can occur in cancer, in vascular diseases, and in many types of inflammatory and immunological processes (26-29). Proteolytic degradation of ECM components by MMPs clearly facilitates carcinoma cell invasion and metastasis. Furthermore, MMPs play important roles in non-matrix bioactive chemokine- and growth-factor processes, and in the modulation of activities of other proteases in cascades (30-32). Interestingly, MMP-9 and MMP-8 have shown tumor-suppressive and defensive effects in breast, skin, and colitis-associated cancer, as well as epithelial-myoepithelial salivary gland and tongue cancer (33-36). With this background, we may assume that oral pathogens and MMPs may play a role in oropharyngeal squamous cell carcinoma (OPSCC). Our aim was to determine the prevalence of R gingipain (Rgp), a key virulence factor of the oral pathogen Pg, and the prevalence of MMP-8 and 9 expression in 202 unselected consecutive OPSCC patients. We further aimed to discover their relationship to our earlier findings regarding HPV status, chymotrypsin-like proteinase of Td (Td-CTLP) immunoexpression, clinical parameters, and patient outcome.

Patients and Methods

Patients and clinicopathological data. The patient cohort originally comprised 331 consecutive patients with oropharyngeal cancer treated at the Helsinki University Hospital between 2000 and 2009, as previously described (37). The series fulfilling the inclusion criteria of this study comprised of 202 patients with treatment-naïve OPSCC treated with curative intention. Included were patients with squamous cell carcinomas (SCC) and subtypes of SCC with the following 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. Excluded from analysis were those patients with palliative intention of treatment (*n*=44), concurrent head and neck squamous cell carcinoma (HNSCC) (*n*=5), earlier treated HNSCC (*n*=11), histology other than SCC (*n*=18), or tumor-tissue unavailability (*n*=52).

The patient- and tumor characteristics of the patient cohort have been reported earlier (7, 37, 38), and appear in Table I as background information. The HPV status classification used here differs from our earlier reported results: earlier classification was based on HPV DNA result whereas our current classification combines results of $p16^{INK4a}$(p16) and HPV mRNA assays as described in detail later in this chapter. Follow-up of all patients was at minimum three years or until death. Survival dates and causes of death came from Statistics Finland. The study received an approval of the Research Ethics Board of the Hospital District of Helsinki and Uusimaa, and an institutional research permission was granted.

Of the 202 patients, 130 had undergone primary surgery. Among these, 116 received additionally either radiotherapy (RT) or chemoradiotherapy (CRT) as adjuvant oncological treatment. Among the 202 patients, 71 received definitive CRT or RT, and of these, 11 underwent additional surgery for residual disease (primary site: 1, neck only: 7, primary site and neck: 3). Tissue samples were collected before RT/CRT from all but two patients, in whom only post-treatment samples were available for immunohistochemistry.

The results on HPV DNA, HPV mRNA and p16 status were available from our earlier analysis (37, 39). HPV status of OPSCC

Table I. *Patient and tumor characteristics stratified by human papillomavirus (HPV) status.*

	All patients n=202 n (%)	HPV-positive n=108 n (%)	HPV-negative n=94 n (%)	*p*-Value	Missing
Age				0.204	
Below 60	115 (57)	66 (61)	49 (52)		
60 and older	87 (43)	42 (39)	45 (48)		
Sex				0.421	
Male	150 (74)	83 (77)	67 (72)		
Female	52 (26)	25 (23)	27 (28)		
Smoking				**<0.001**	31
Never	26 (15)	24 (28)	2 (2)		
Earlier	49 (29)	37 (44)	12 (14)		
Currently	96 (56)	24 (28)	72 (84)		
Excess alcohol consumption				**<0.001**	79
Never	61 (50)	39 (70)	22 (33)		
Earlier	24 (20)	7 (12)	17 (25)		
Currently	38 (30)	10 (18)	28 (42)		
T class				0.573	
T1-T2	114 (56)	63 (58)	51 (54)		
T3-T4	88 (44)	45 (42)	43 (46)		
N class				**0.049**	
N0	39 (19)	15 (14)	24 (26)		
N1-N3	163 (81)	93 (86)	70 (74)		
Tumor stage				**0.018**	
I-II	30 (15)	10 (9)	20 (21)		
III-IV	172 (85)	98 (91)	74 (79)		
Tumor grade				**<0.001**	
1	18 (9)	4 (4)	14 (15)		
2	78 (39)	33 (31)	45 (48)		
3	106 (52)	71 (66)	35 (37)		
Tumor site				**0.047**	
Anterior wall of oropharynx	61 (30)	26 (24)	35 (37)		
Lateral wall of oropharynx	117 (58)	79 (73)	38 (40)		
Posterior wall of oropharynx	3 (1)	1 (1)	2 (2)		
Superior wall of oropharynx	21 (10)	2 (2)	19 (20)		

T class, primary tumor size; N class, presence of regional lymph node metastasis. Statistically significant *p*-Values are shown in bold.

was determined according to a classification method originally proposed by Smeets *et al*. (40) and later modified by our research group (39). The Smeets *et al*. classification method combines p16 and HPV DNA test results, whereas in our modified classification method, the HPV DNA result is replaced by the HPV mRNA result. We have previously shown that ISH for high-risk HPV E6/E7 mRNA is a highly specific and sensitive method for detecting HPV in OPSCC (39). The samples were divided into an HPV-positive group consisting of 108 tumors that included only p16-positive and HPV mRNA-positive samples. HPV-negative group included 94 either p16-positive but HPV mRNA-negative samples, or p16-negative and HPV mRNA-negative samples, or p16-negative but HPV mRNA-positive samples. Furthermore, data on *Treponema denticola* chymotrypsin-like protease (Td-CTLP) immunoexpression has appeared earlier (7).

Immunohistochemistry for gingipain and matrix metalloproteinases 8 and 9. We prepared tissue microarray (TMA) blocks and immunostained slides as described earlier (41). The immunohistochemical staining for Rgp was performed with polyclonal rabbit antibody for *Porphyromonas gingivalis* GingipainR1 (1: 800, Biorbyt Ltd., Cambridge, UK), with polyclonal rabbit anti-human MMP-8 (42, 43) for MMP-8, and with monoclonal mouse anti-human MMP-9 IgG (1:1000, IIA5, NeoMarkers Inc., Thermo Fisher Scientific, Cheshire, UK) for MMP-9.

Immunohistochemical scoring. The TMA slides immunostained with Rgp, MMP-8, and 9 antibody were scored by two researchers (J.H. and A.K.K.) separately, at that stage blinded to the clinical data. Any discordance in scoring was solved by reassessment in order to achieve consensus. Rgp and MMP-9 scoring in tumor tissue was based on intensity of positivity: none (0), mild (1), moderate (2), or strong (3). Rgp scoring in inflammatory cells and MMP-8 and MMP-9 scoring in neutrophils was assessed based on the number of positive cells as follows: negative (0), 1-20 positive cells (1), 20-100 positive cells (2) and >100 positive cells (3).

Statistical analysis. Statistical analysis was performed using SPSS version 27.0 (IBM SPSS Statistics, IBM Corporation, New York, NY, USA), R version 4.0.3 (Foundation for Statistical Computing,

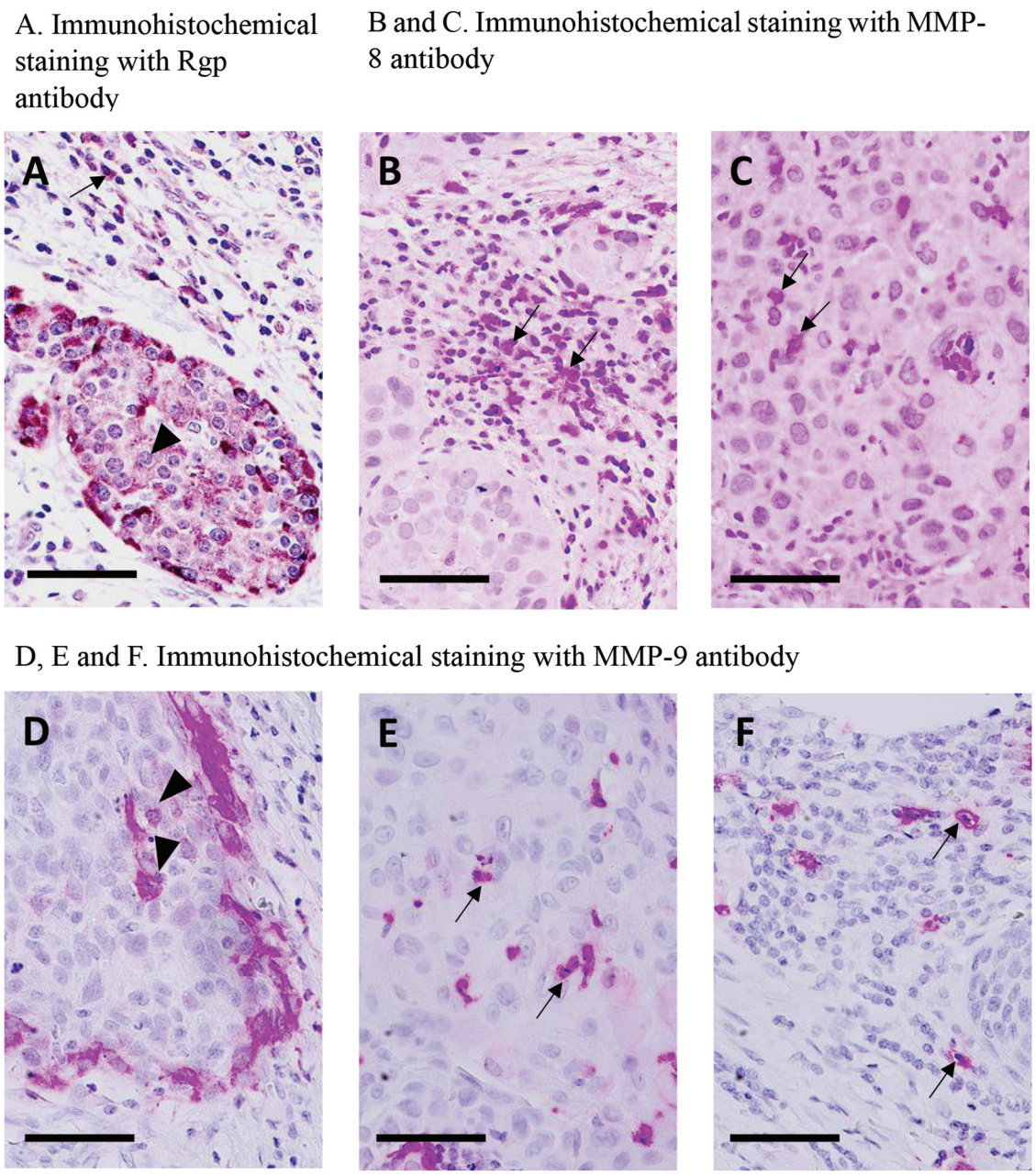

Figure 1. *Immunohistochemical staining of oropharyngeal squamous cell carcinoma (OPSCC) samples with R gingipain (Rgp) antibody specific to Porphyroromonas gingivalis (Pg), matrix metalloproteinase 8 (MMP-8) antibody and 9 (MMP-9) antibody. OPSCC with positive immunoexpression of Rgp in tumor and inflammatory cells of stroma (A), MMP-8 expression in inflammatory cells of stroma (B) and inflammatory cells infiltrating the tumor (C), MMP-9 expression in tumor cells (D), inflammatory cells infiltrating the tumor (E), and inflammatory cells of stroma (F). Arrowheads indicate tumor tissue, and arrows indicate inflammatory cells. Scale bar length, 100 μm. Magnification, ×400.*

Vienna, Austria) and STATA/MP (version 16.1, StataCorp LLC, College Station, TX, USA). Statistical differences between categorical variables were evaluated by Fisher's exact test or the Fisher-Freeman-Halton exact test, and between ordinal variables using the Linear-by-linear association test. The measure of association between ordinal variables was evaluated by Spearman correlation coefficients with 95% confidence limits. Correlations with Spearman rho value below and equal to 0.3 were regarded as negligible.

The Cox proportional hazards model served in univariable and multivariable survival analysis. In the analysis, a competing event with death by OPSCC was death by other cause, and sub distribution hazard ratios (SHR) were calculated. The Cox regression assumption of constant hazard ratios over time was assessed with the Schoenfeld

Table II. *Biomarker associations among 202 consecutive oropharyngeal squamous cell carcinoma (OPSCC) patients stratified according to human papillomavirus (HPV) status.*

	All patients n=202 n (%)	HPV-positive n=108 n (%)	HPV-negative n=94 n (%)	*p*-Value	Missing
Rgp in tumor				**0.001**	9
Score 0	38 (20)	27 (25)	11 (12)		
1	58 (30)	31 (30)	27 (31)		
2	62 (32)	39 (37)	23 (26)		
3	35 (18)	8 (8)	27 (31)		
Rgp in inflammatory cells				0.561	9
Score 0	56 (29)	33 (31)	23 (26)		
1	44 (22)	22 (21)	22 (25)		
2	74 (38)	41 (39)	33 (38)		
3	19 (10)	9 (9)	10 (11)		
MMP-8 in tumor neutrophils				**0.011**	6
Score 0	71 (36)	48 (46)	23 (25)		
1	67 (34)	32 (30)	35 (38)		
2	54 (28)	22 (21)	32 (35)		
3	4 (2)	3 (3)	1 (1)		
MMP-8 in stroma neutrophils				0.340	4
Score 0	13 (7)	7 (7)	6 (6)		
1	73 (37)	45 (43)	28 (30)		
2	81 (41)	36 (34)	45 (48)		
3	31 (16)	17 (16)	14 (15)		
MMP-9 in tumor				0.076	4
Score 0	168 (85)	93 (89)	75 (81)		
1	26 (13)	11 (10)	15 (16)		
2	2 (1)	1 (1)	1 (1)		
3	2 (1)	0	2 (2)		
MMP-9 in neutrophils				**0.011**	4
Score 0	16 (8)	12 (11)	4 (4)		
1	88 (44)	54 (51)	34 (37)		
2	78 (39)	30 (29)	48 (52)		
3	16 (8)	9 (9)	7 (7)		

Rgp, Immunoexpression of R gingipain in tumor cells and in inflammatory cells; MMP-8, immunoexpression of matrix metalloproteinase 8; MMP-9, immunoexpression of matrix metalloproteinase 9. Statistically significant *p*-Values are shown in bold.

residuals plotted over time, as well as testing for trend. No significant deviations from the assumption were observed.

The disease-specific survival (DSS) was presented with cumulative incidence function (cumulative death rates over time) in Aalen-Johansen plots considering the other unrelated deaths to OPSCC. Grey's test served for statistical significance between the categories. The follow-up time in the DSS evaluation was defined as the period between the last treatment day and the last day of follow-up or date of death from the disease.

Results

Rgp, MMP-8 and MMP-9 were immunoexpressed in OPSCC and inflammatory cells. The immunoexpression of Rgp was cytoplasmic in carcinoma cells (Figure 1A). In addition, Rgp immunopositivity was detectable in endothelial cells, neutrophils, and lymphocytes. Rgp-immunopositive inflammatory cells were present in the stroma only. We scored Rgp immunopositivity both in carcinoma cells and in inflammatory cells. Of the 193 samples available for Rgp staining, Rgp was immunoexpressed in tumor cells in 155 (80%), and in inflammatory cells in 137 (71%) (Table II).

The MMP-8 immunopositivity was negative in tumor cells and detectable only in neutrophils within and surrounding the tumor; we scored it separately for each location (Figure 1B and C). MMP-8 immunoexpression occurred in tumor neutrophils in 63% (125 of 196) of the tumor samples available for MMP-8 staining, and in stroma neutrophils in 93% (185 of 198) of the stroma samples available for MMP-8 staining (Table II).

The immunoexpression of MMP-9 was cytoplasmic in carcinoma cells (Figure 1D). In addition, MMP-9 immunopositivity was detectable in neutrophils within the carcinoma and in the surrounding stroma (Figure 1E and F). We scored Rgp immunopositivity both in carcinoma cells and in inflammatory cells, the latter including both tumoral and stromal neutrophils. MMP-9 was immunoexpressed,

Table III. *Biomarker correlations among 202 consecutive oropharyngeal squamous cell carcinoma (OPSCC) patients.*

	Total n	Rgp in tumor				Rgp in inflammatory cells			
		Correlation coefficient Spearman rho	Confidence		*p*-Value	Correlation coefficient Spearman rho	Confidence		*p*-Value
			Lower	Upper			Lower	Upper	
Rgp in tumor	193								
Rgp in inflammatory cells	193	0.352	0.218	0.473	**<0.001**				
MMP-8 in tumor neutrophils	196	0.092	–0.055	0.235	0.207	0.067	–0.08	0.212	0.357
MMP-8 in stroma neutrophils	198	0.067	–0.081	0.211	0.362	0.084	–0.064	0.227	0.252
MMP-9 in tumor	198	0.279	0.138	0.408	**<0.001**	0.135	–0.011	0.276	0.062
MMP-9 in neutrophils	198	0.096	–0.051	0.238	0.188	0.043	–0.103	0.188	0.552
Td-CTLP in tumor	201	0.039	–0.107	0.184	0.589	–0.053	–0.197	0.094	0.468
HPV status	202	0.23	0.088	0.363	**0.001**	0.044	–0.102	0.188	0.544

	Total n	MMP-8 in tumor neutrophils				MMP-8 in stroma neutrophils			
		Correlation coefficient Spearman rho	Confidence		*p*-Value	Correlation coefficient Spearman rho	Confidence		*p*-Value
			Lower	Upper			Lower	Upper	
Rgp in tumor	193								
Rgp in inflammatory cells	193								
MMP-8 in tumor neutrophils	196								
MMP-8 in stroma neutrophils	198	0.486	0.367	0.589	**<0.001**				
MMP-9 in tumor	198	0.181	0.037	0.317	**0.011**	0.043	–0.102	0.186	0.552
MMP-9 in neutrophils	198	0.557	0.448	0.649	**<0.001**	0.695	0.613	0.763	**<0.001**
Td-CTLP in tumor	201	0.221	0.079	0.355	**0.002**	0.112	–0.033	0.251	0.119
HPV status	202	0.197	0.055	0.332	**0.006**	0.088	–0.056	0.229	0.218

	Total n	MMP-9 in tumor				MMP-9 in neutrophils			
		Correlation coefficient Spearman rho	Confidence		*p*-Value	Correlation coefficient Spearman rho	Confidence		*p*-Value
			Lower	Upper			Lower	Upper	
Rgp in tumor	193								
Rgp in inflammatory cells	193								
MMP-8 in tumor neutrophils	196								
MMP-8 in stroma neutrophils	198								
MMP-9 in tumor	198								
MMP-9 in neutrophils	198	0.063	–0.082	0.204	0.38				
Td-CTLP in tumor	201	0.139	–0.004	0.278	0.051	0.192	0.05	0.327	**0.007**
HPV status	202	0.113	–0.031	0.252	0.113	0.203	0.062	0.337	**0.004**

Rgp, Immunoexpression of R gingipain; Td-CTLP, immunoexpression of Treponema denticola chymotrypsin-like protease; HPV status, Human papillomavirus status; MMP8, immunoexpression of matrix metalloproteinase 8; MMP9, immunoexpression of matrix metalloproteinase 9. Statistically significant *p*-Values are shown in bold. Uncategorized scores were used.

among 198 samples available for MMP-9 staining, in tumor cells in 30 (15%), and in neutrophils in 182 (92%) (Table II).

Neither Rgp, MMP-8, nor MMP-9 showed any correlation exceeding our arbitrary limit for low correlations (correlation coefficient, rho >0.3) with patient or tumor characteristics (data not shown).

Rgp, MMP-8 and MMP-9 showed low correlation with HPV status of OPSCC. Rgp in tumor cells, MMP-8 in tumor neutrophils and MMP-9 in neutrophils correlated with tumor HPV status, albeit the correlation was low (Table III). In HPV-positive disease, mild immunoexpression of Rgp in tumor cells was more common, whereas in HPV-negative disease, strong immunoexpression dominated. A similar trend applied for MMP-8 in tumor neutrophils and MMP-9 in neutrophils (Table II). Neither Rgp, MMP-8, nor MMP-9 showed any correlation exceeding our arbitrary limit for low correlations (correlation coefficient, rho >0.3) with Td-CTLP (Table III).

Table IV. *Biomarker correlations among 108 human papillomavirus (HPV) positive oropharyngeal squamous cell carcinoma (OPSCC) patients.*

		Rgp in tumor				Rgp in inflammatory cells			
	Total n	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value
Rgp in tumor	105								
Rgp in inflammatory cells	105	0.474	0.305	0.614	**<0.001**				
MMP-8 in tumor neutrophils	103	0.116	–0.085	0.308	0.242	–0.034	–0.231	0.167	0.737
MMP-8 in stroma neutrophils	103	0.056	–0.145	0.252	0.577	0.053	–0.147	0.250	0.592
MMP-9 in tumor	103	0.206	0.007	0.389	**0.037**	0.183	–0.016	0.369	0.064
MMP-9 in neutrophils	103	0.094	–0.107	0.288	0.344	0.053	–0.148	0.249	0.595
Td-CTLP in tumor	105	–0.108	–0.214	0.180	0.857	–0.054	–0.248	0.145	0.586

		MMP-8 in tumor neutrophils				MMP-8 in stroma neutrophils			
	Total n	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value
Rgp in tumor	105								
Rgp in inflammatory cells	105								
MMP-8 in tumor neutrophils	103								
MMP-8 in stroma neutrophils	103	0.426	0.250	0.575	**<0.001**				
MMP-9 in tumor	103	0.147	–0.052	0.336	0.136	0.005	–0.194	0.203	0.962
MMP-9 in neutrophils	103	0.462	0.291	0.605	**<0.001**	0.703	0.587	0.791	**<0.001**
Td-CTLP in tumor	105	0.258	0.064	0.433	**0.008**	0.149	–0.050	0.336	0.130

		MMP-9 in tumor				MMP-9 in neutrophils			
	Total n	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value
Rgp in tumor	105								
Rgp in inflammatory cells	105								
MMP-8 in tumor neutrophils	103								
MMP-8 in stroma neutrophils	103								
MMP-9 in tumor	103								
MMP-9 in neutrophils	103	0.026	–0.172	0.222	0.796				
Td-CTLP in tumor	105	0.025	–0.173	0.221	0.798	0.179	–0.019	0.363	0.068

Rgp, Immunoexpression of R gingipain; Td-CTLP, immunoexpression of Treponema denticola chymotrypsin-like protease; MMP8, immunoexpression of matrix metalloproteinase 8; MMP9, immunoexpression of matrix metalloproteinase 9. Statistically significant *p*-Values are shown in bold. Uncategorized scores were used.

Among biomarkers Rgp, MMP-8 and MMP-9, several statistically significant correlations did emerge. The biomarker correlations among all patients and among HPV-positive disease group were similar (Table III and Table IV). HPV-negative disease group differed from these in respect of tumoral Rgp correlations: tumoral Rgp correlated with tumoral MMP-9 (rho=0.315; p=0.003), and not with Rgp in inflammatory cells (Table V).

Tumor MMP-9 and Rgp in inflammatory cells were risk factors for survival. In survival analysis, we evaluated the score groups separately and dichotomized into the categories presented in Table VI and Table VII. In univariable survival analysis of all 202 patients, tumoral MMP-9 presented as a risk factor for poor DSS when we included death by cause other than OPSCC as a competing factor in the Cox proportional hazard model. Univariable analysis showed Rgp in tumor cells or inflammatory cells, MMP-8 in tumor or in stroma neutrophils, and MMP-9 in neutrophils to remain statistically non-significant factors.

In multivariable analysis adjusted for known patient and tumor characteristics, and for available biomarkers, in addition

Table V. *Biomarker correlations among 94 human papillomavirus (HPV) negative oropharyngeal squamous cell carcinoma (OPSCC) patients.*

		Rgp in tumor				Rgp in inflammatory cells			
	Total n	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value
Rgp in tumor	88								
Rgp in inflammatory cells	88	0.219	0.004	0.415	**0.040**				
MMP-8 in tumor neutrophils	87	–0.027	–0.243	0.190	0.801	0.186	–0.032	0.387	0.051
MMP-8 in stroma neutrophils	87	0.043	–0.175	0.257	0.692	0.118	–0.101	0.326	0.277
MMP-9 in tumor	88	0.315	0.106	0.496	**0.003**	0.084	–0.134	0.294	0.435
MMP-9 in neutrophils	88	0.028	–0.188	0.242	0.794	0.017	–0.199	0.232	0.872
Td-CTLP in tumor	87	–0.096	–0.306	0.123	0.378	–0.079	–0.291	0.140	0.467

		MMP-8 in tumor neutrophils				MMP-8 in stroma neutrophils			
	Total n	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value
Rgp in tumor	88								
Rgp in inflammatory cells	88								
MMP-8 in tumor neutrophils	87								
MMP-8 in stroma neutrophils	87	0.543	0.374	0.677	**<0.001**				
MMP-9 in tumor	88	0.180	–0.034	0.377	0.089	0.068	–0.145	0.274	0.522
MMP-9 in neutrophils	88	0.634	0.488	0.746	**<0.001**	0.666	0.529	0.769	**<0.001**
Td-CTLP in tumor	87	0.031	–0.183	0.243	0.771	–0.006	–0.217	0.205	0.953

		MMP-9 in tumor				MMP-9 in neutrophils			
	Total n	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value	Correlation coefficient Spearman rho	Confidence Lower	Confidence Upper	*p*-Value
Rgp in tumor	88								
Rgp in inflammatory cells	88								
MMP-8 in tumor neutrophils	87								
MMP-8 in stroma neutrophils	87								
MMP-9 in tumor	88								
MMP-9 in neutrophils	88	0.057	–0.154	0.263	0.588				
Td-CTLP in tumor	87	0.181	–0.031	0.377	0.084	0.063	–0.150	0.270	0.554

Rgp, Immunoexpression of R gingipain; Td-CTLP, immunoexpression of Treponema denticola chymotrypsin-like protease; MMP8, immunoexpression of matrix metalloproteinase 8; MMP9, immunoexpression of matrix metalloproteinase 9. Statistically significant *p*-Values are shown in bold. Uncategorized scores were used.

to tumoral MMP-9, Rgp in inflammatory cells influenced the DSS (p=0.008 and 0.021, respectively). Tumoral MMP-9 worsened the DSS (SHR=2.4) and Rgp in inflammatory cells improved it (HR=0.5) (Table VI, Figure 2).

Tumor MMP-9 in combination with Rgp in inflammatory cells as a prognostic factor. Further, we investigated whether the combination of tumoral MMP-9 and Rgp in inflammatory cells would provide additional characterization of the patients. This combination included three categories: positive tumoral MMP-9 (scores 1-3) combined with any immunoexpression of Rgp in inflammatory cells (scores 0-3); negative tumoral MMP-9 (score 0) combined with low immunoexpression of Rgp in inflammatory cells (scores 0-1); and negative tumoral MMP-9 (score 0) combined with high immunoexpression of Rgp in inflammatory cells (scores 2-3). Indeed, the group with high Rgb in their inflammatory cells and negative tumoral MMP-9 seemed to show better survival than did patients with other combinations (Table VI, Figure 2).

Survival in the HPV-negative subgroup. Among HPV-negative OPSCC patients, tumoral MMP-9, Rgp in inflammatory cells,

Table VI. *Univariable and multivariable Cox regression analysis for Disease-Specific survival (DSS) in a series of all 202 oropharyngeal squamous-cell-carcinoma (OPSCC) patients. Death by cause other than OPSCC is included in the analysis as a competing factor.*

	Univariable analysis All patients			Multivariable analysis* All patients			Multivariable analysis** All patients		
	SHR	95% CI	*p*-Value	SHR	95% CI	*p*-Value	SHR	95% CI	*p*-Value
Age at time of diagnosis	1.0	1.0-1.0	0.408	1.0	1.0-1.1	0.420	1.0	1.0-1.1	0.327
Sex									
Female *vs*. male	0.5	0.2-1.1	0.071	0.4	0.1-0.9	**0.038**	0.4	0.2-1.0	**0.046**
T class									
T3-4 *vs*. T1-2	1.5	0.9-2.7	0.132	1.3	0.7-2.4	0.407	1.3	0.7-2.4	0.499
N class									
N1-3 *vs*. N0	1.8	0.8-4.2	0.149	2.4	1.0-6.0	0.058	2.1	0.9-4.9	0.108
Tumor grade	1								
Gr2 *vs*. Gr1	1.2	0.4-3.2	0.747						
Gr3 *vs*. Gr1	0.5	0.2-1.4	0.207						
Smoking									
Yes *vs*. no	2.4	1.2-4.6	**0.010**	1.6	0.8-3.4	0.173	1.8	0.9-3.8	0.122
Smoking	1								
Earlier *vs*. never	1.6	0.4-6.1	0.477						
Currently *vs*. never	3.3	1.0-11.2	0.051						
HPV status									
HPV-negative *vs*. HPV-positive	2.7	1.5-4.8	**0.001**	1.9	0.9-3.8	0.078	2.1	1.0-4.4	**0.043**
Rgp in tumor									
1-3 *vs*. 0	2.8	1.0-7.9	0.050	3.4	1.0-11.8	0.054			
Rgp in inflammatory cells									
2-3 *vs*. 0-1	0.6	0.3-1.1	0.077	0.5	0.2-0.9	**0.021**			
Td-CTLP in tumor	1								
1	1.4	0.6-3.3	0.401						
2	1.4	0.6-3.3	0.420						
3	3.0	1.0-8.9	**0.046**						
MMP-9 in tumor									
1-3 *vs*. 0	2.1	1.1-4.1	**0.025**	2.4	1.2-4.4	**0.008**			
MMP-9 in neutrophils									
2-3 *vs*. 0-1	1.7	1.0-3.0	0.068						
MMP-8 in tumor neutrophils									
2-3 *vs*. 0-1	1.3	0.7-2.3	0.445						
MMP-8 in stroma neutrophils									
2-3 *vs*. 0-1	1.0	0.6-1.7	0.942						
Combination									
Tumor MMP-9 and Rgp in neutrophils									
0 MMP-9=0, Rgp=2-3	1.0						1.0		
1 MMP-9=0, Rgp=0-1	2.2	1.1-4.7	**0.034**				2.4	1.1-5.5	**0.035**
2 MMP-9=1-3, Rgp=0-1, 2-3	3.5	1.5-8.2	**0.004**				4.3	1.8-10.4	**0.001**

T class, Primary tumor size; N class, presence of regional lymph node metastasis; HPV status, Human papillomavirus status; Rgp, immunoexpression of R gingipain; Td-CTLP, immunoexpression of Treponema denticola chymotrypsin-like protease; MMP8, immunoexpression of matrix metalloproteinase 8; MMP9, immunoexpression of matrix metalloproteinase 9; Combination, immunoexpression of MMP9 in tumor cells in combination with Rgp in inflammatory cells; SHR, sub distribution hazard rate. Statistically significant *p*-Values are shown in bold. *Multivariable Cox regression analysis for disease specific survival adjusted for age, sex, T-class, N class, smoking, and HPV status, including Rgp in tumor, Rgp in inflammatory cells and MMP-9 in tumor as variables. **Multivariable Cox regression analysis for disease specific survival adjusted for age, sex, T-class, N class, smoking, and HPV status, including combination MMP-9 in tumor and Rgp in inflammatory cells as a variable.

and their combination emerged in univariable analysis as significant factors affecting the DSS (Table VII) in a similar fashion as they did among all patients. In multivariable analysis adjusted for known patient- and tumor characteristics, MMP-9 in neutrophils additionally worsened the DSS (Table VII).

Survival in the HPV-positive subgroup. In univariable analysis of HPV-positive OPSCC, risk factors for poor DSS were high age and low tumor grade, as previously reported (37, 44). Neither Rgp, MMP-8, nor MMP-9 showed any statistical significance regarding DSS (data not shown).

Table VII. *Univariable and multivariable Cox regression analysis for Disease-Specific survival (DSS) among 94 human papillomavirus (HPV) negative oropharyngeal squamous cell carcinoma (OPSCC) patients. Death by cause other than OPSCC is included in analysis as a competing factor.*

	Univariable analysis HPV-negative patients			Multivariable analysis* HPV-negative patients			Multivariable analysis** HPV-negative patients		
	SHR	95% CI	*p*-Value	SHR	95% CI	*p*-Value	SHR	95% CI	*p*-Value
Age at time of diagnosis	1.0	0.9-1.0	0.144	1.0	0.9-1.0	0.450	1.0	0.9-1.0	0.666
Sex									
Female *vs*. male	0.5	0.2-1.3	0.176	0.4	0.1-1.0	**0.049**	0.4	0.1-1.1	0.087
T class									
T3-4 *vs*. T1-2	1.1	0.6-2.3	0.681	1.0	0.4-2.3	0.997	0.8	0.4-1.8	0.631
N class									
N1-3 *vs*. N0	1.8	0.8-4.1	0.181	2.0	0.8-4.9	0.129	2.3	0.9-5.7	0.079
Tumor grade	1.0								
Gr2 *vs*. Gr1	2.0	0.6-7.1	0.288						
Gr3 *vs*. Gr1	1.5	0.4-5.8	0.535						
Smoking									
Yes *vs*. no	6.6	0.9-49.4	0.065	4.9	0.7-34.9	0.115	5.5	0.7-41.4	0.101
Rgp in tumor									
1-3 *vs*. 0	2.2	0.5-9.5	0.273						
Rgp in inflammatory cells									
2-3 *vs*. 0-1	0.5	0.2-1.0	**0.042**	0.4	0.2-0.9	**0.022**			
Td-CTLP in tumor	1								
1	1.0	0.3-3.7	0.994						
2	0.9	0.2-3.3	0.879						
3	1.3	0.3-5.4	0.764						
MMP9 in tumor									
1-3 *vs*. 0	2.3	1.1-4.9	**0.028**	3.5	1.7-7.3	**0.001**			
MMP-9 in neutrophils									
2-3 *vs*. 0-1	2.0	0.9-4.3	0.085	2.6	1.1-6.3	**0.033**			
MMP-8 in tumor neutrophils									
2-3 *vs*. 0-1	1.5	0.8-3.1	0.222						
MMP-8 in stroma neutrophils									
2-3 *vs*. 0-1	1.1	0.5-2.3	0.794						
Combination									
Tumor MMP-9 and Rgp in neutrophils									
0 MMP-9=0, Rgp=2-3	1						1		
1 MMP-9=0, Rgp=0-1	3.1	1.1-8.7	**0.031**				3.4	1.2-9.8	**0.024**
2 MMP-9=1-3, Rgp=0-1, 2-3	4.9	1.6-14.6	**0.005**				6.5	2.0-20.6	**0.001**

T class, Primary tumor size; N class, presence of regional lymph node metastasis; Rgp, immunoexpression of R gingipain; Td-CTLP, immunoexpression of Treponema denticola chymotrypsin like protease; MMP8, immunoexpression of matrix metalloproteinase 8; MMP9, immunoexpression of matrix metalloproteinase 9; Combination, immunoexpression of MMP9 in tumor cells in combination with Rgp in inflammatory cells; SHR, sub distribution hazard rate. Statistically significant *p*-Values are shown in bold. *Multivariable Cox regression analysis for disease specific survival adjusted for age, sex, T-class, N class, and smoking, including Rgp in tumor, Rgp in inflammatory cells and MMP-9 in tumor as variables. **Multivariable Cox regression analysis for disease specific survival adjusted for age, sex, T-class, N class, and smoking, including combination MMP-9 in tumor and Rgp in inflammatory cells as a variable.

Discussion

Here, we show the occurrence of Rgp—a key virulence factor specific to the oral pathobiont Pg—in both HPV-positive and HPV-negative OPSCC. Tumoral Rgp and tumoral MMP-9 correlated positively in HPV-negative OPSCC, but no correlation existed in HPV-positive OPSCC. Our results suggest that, in OPSCC, tumoral MMP-9 may be related to poor outcome, especially in HPV-negative disease, whereas among these same groups, Rgp in inflammatory cells improves the DSS.

We found cytoplasmic Rgp immunoexpression in OPSCC cells, whereas Gao *et al*. additionally detected anti-Pg and anti-lysine gingipain (Kgp) in cell nuclei, in esophageal squamous cell carcinoma (ESCC) (45). This may reflect differences between these cancers or between the antibodies

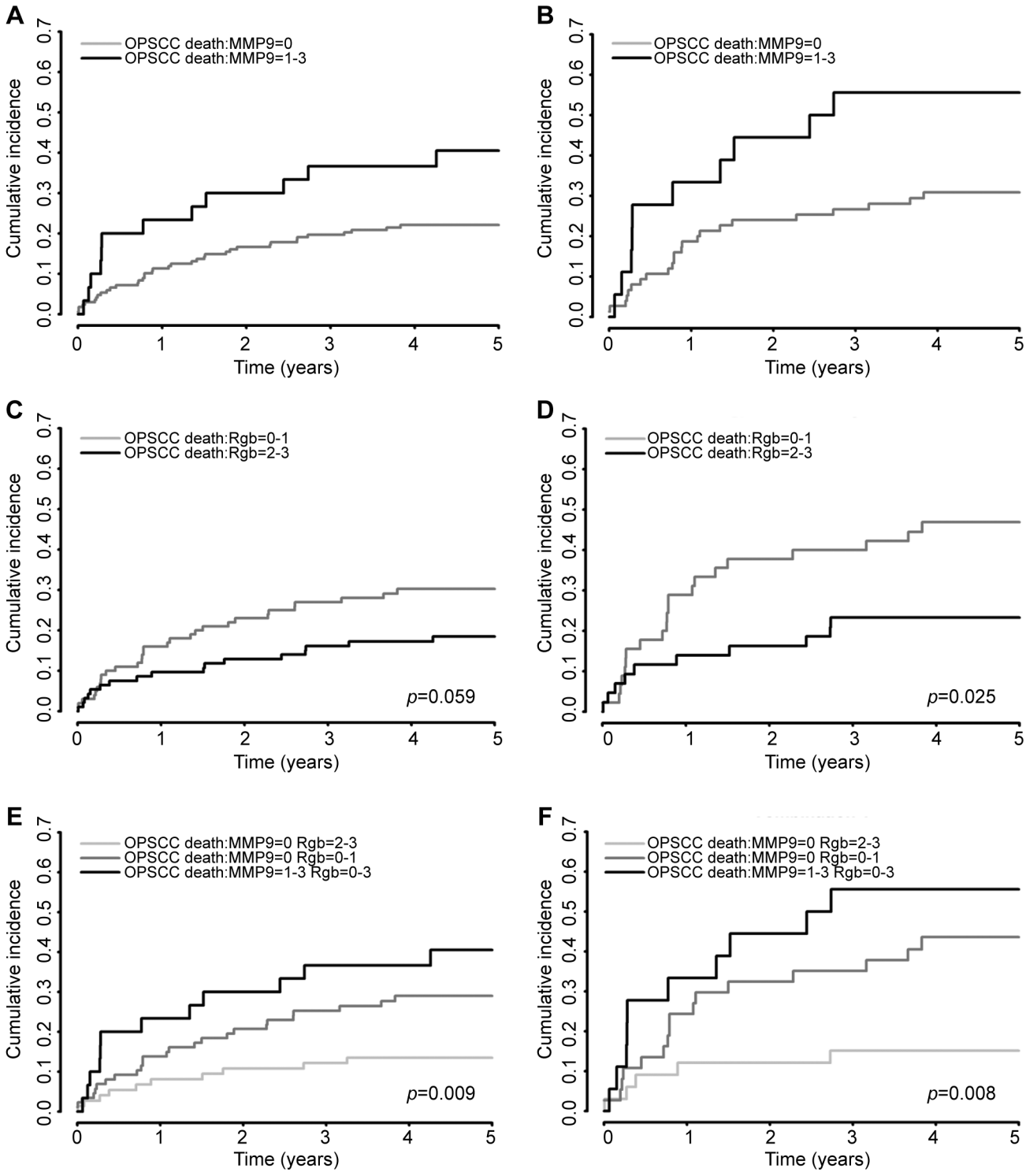

Figure 2. *The disease specific cumulative incidence (cumulative death rates over time) presented as Aalen-Johansen plots. Deaths from causes other than OPSCC were included in the analysis but are not shown in the figure for simplicity. In total there were 26 (16 in HPV-negative group) deaths unrelated to OPSCC. The cumulative incidence function did not statistically differ between the groups for deaths unrelated to OPSCC. Gray's test assessed differences between groups; R gingipain (Rgp) in inflammatory cells, tumoral matrix metalloproteinase 9 (MMP-9), and combination (tumoral MMP-9 and Rgp in inflammatory cells) among all patients and among HPV-negative oropharyngeal squamous cell carcinoma (OPSCC) patients. Tumoral MMP-9 among all patients (A), and in HPV-negative disease (B), Rgp in inflammatory cells among all patients (C), and in HPV-negative disease (D), and combination of tumoral MMP-9 and Rgp in inflammatory cells among all patients (E) and in HPV-negative disease (F).*

used. In our patients, Rgp was also present in inflammatory cells in the tumor stroma and infiltrating the tumor. In periodontal tissue, Pg frequently co-exists with Td (11). This is apparent also in our patient cohort, because we earlier detected Td-specific Td-CTLP immunoexpression in the same OPSCC samples (7). No statistically significant correlation with tumoral Rgp and Td-CTLP was, however, present in our current study.

Pg can invade oral epithelial and endothelial cells and induce proinflammatory cytokine- and MMP production, as well as proMMP and plasminogen activation (24, 46). This can eventually promote tumorigenic microenvironment modifications of the cellular environment both intra- and extracellularly. Accordingly, in our patient samples, we detected Rgp in the OPSCC cytoplasm and endothelial cells. Based on our studies, it is impossible, however, to assess whether host-cell invasion of Pg occurred, or whether this occurred before or after these cells' malignant transformation.

We detected MMP-9 in tumor tissue in 15% of the OPSCCs, and in neutrophils in 92% of the OPSCCs; this was evident in both HPV-positive and negative disease. Earlier, elevated MMP-9 expression have been detected in oral tongue squamous cell carcinoma (OTSCC) (47), and in OSCC (48). Interestingly, elevated levels of MMP-9 in serum (49) and in saliva (50, 51) have been associated with OSCC. Furthermore, overexpression of MMP-9 has been evident in Pg-infected human oral epithelial cells, human gingival keratinocytes (52, 53), murine model cells (10, 54) and OSCC cell lines (17, 55). We observed a statistically significant low positive correlation between tumoral MMP-9 and tumoral Rgp in HPV-negative OPSCC, but this correlation was negligible (rho<0.3) in the whole patient cohort, and in HPV-positive disease. Rgp showed no statistically significant correlation with MMP-9 in neutrophils.

We earlier showed Td-CTLP to be present in oropharyngeal and orodigestive tumor tissues, and in *in vitro* conversion of proMMP-8 and -9 to their active forms by Td-CTLP (7, 8). Although in our current patient cohort, we did not find statistically significant correlation between Td-CTLP and MMP-9 in tumor cells or in inflammatory cells, Td-CTLP, among other factors, may not be ruled out as having some influence on MMP-9 expression.

In survival analysis, tumoral MMP-9 was an independent prognostic factor for poor DSS in OPSCC among all patients and in those with HPV-negative disease, which is in line with the reported MMP-9 association with poor prognosis in OTSCC (56), gastric cancer (57), breast cancer (58), colorectal cancer (59), and non-small cell lung cancer (60). Our result supports the idea that the carcinogenesis in HPV-negative OPSCC resembles more that of the OSCC, and it is different from virus-driven carcinogenesis of HPV-positive disease, in regard to the involvement of MMP-9. Evidence differs, however, as to MMP-9 acting as a suppressor in cancer: Bendrik *et al*. (33) evidenced that overexpression of MMP-9 caused tumor regression and decreased angiogenesis in murine model and Luukkaa *et al*. (35) detected higher MMP-9 index to predict better survival *in vitro* in epithelial-myoepithelial salivary gland cancer. In colitis associated cancer, MMP-9 played a protective role as evidenced *in vivo* using MMP-9 knock-out mice and *in vitro* enterocyte cell line (34).

Tumoral Rgp immunoexpression did not reach statistical significance (p=0.054) as an independent prognostic factor for DSS in our patient cohort. This differs from the findings of Ahn *et al*. (4) revealing Pg as being a prognostic marker for survival in orodigestive cancer independent of periodontitis, and Gao *et al*. (45) reporting a positive correlation between Pg infection and overall survival rate in ESCC.

Rgp immunoreactivity in inflammatory cells, however, played a prognostic role among all patients in a multivariable setting and in HPV-negative disease both in univariable and multivariable settings. Interestingly, Rgp in inflammatory cells seemed to improve prognosis. This may be explained by phagocytosis of Pg, and as such a manifestation of an efficient immune-response, or by some Pg-induced defensive inflammatory response in the tumor microenvironment promoting, at least to some degree, tumor suppression. As with tumor MMP-9, Rgp in inflammatory cells was not a statistically significant prognostic factor in HPV-positive OPSCC. This may be due to low event rate in HPV-positive tumors, or may account to the differences in etiology, and therefore, in carcinogenesis of the HPV-positive and negative OPSCC. The results of survival analysis additionally confirmed our earlier reported factors, HPV status and Td-CTLP, as risk factors when a competing factor was included in the model.

As a limitation to our study, we collected data retrospectively on non-randomized patients, making our results susceptible to unknown biases. The patient cohort included a consecutive series of all OPSCC patients treated over a 10-year period with curative intent at our institute. The patient history available was limited, especially regarding details on smoking and alcohol abuse.

In our survival analysis, we did, however, include combinations of biomarkers to provide further insight to our observations. We also used sub distribution hazard ratios and the cumulative incidence function, methods appropriate for prognostic studies, to account for the bias caused by deaths unrelated to OPSCC, as these accounted for approximately 35% of all deaths encountered, precluding the occurrence of OPSSC related deaths. In survival analysis, we observed, that for the combination factor of tumor MMP-9 and Rgb in inflammatory cells, the MMP-9 component of the combination impaired prognosis, but the inflammatory-cell Rgp component of the same combination improved it. Examined altogether, this combination supports our

observations concerning separate biomarkers, as well as earlier findings by other researchers (45, 61), as to the role of MMP-9 in impaired prognosis.

The possible role of Rgp in carcinogenesis of OPSCC may be speculated upon, because at least two mechanisms seem possible: a tumorigenic role as a promoter and activator of MMP-9, and an anti-tumorigenic role as a promoter of inflammatory-cell response tumor-suppressive properties. Regardless, considering our limited sample size, we must avoid strong conclusions concerning survival results.

Conclusion

In short, Rgp was present in both HPV-positive and HPV-negative OPSCC. A positive correlation existed between tumoral Rgp and tumoral MMP-9 in HPV-negative OPSCC, but not in HPV-positive OPSCC. Tumoral MMP-9 may be related to poor DSS in OPSCC, especially among patients with HPV-negative disease, whereas Rgp in inflammatory cells improved DSS in these same groups. The role of Rgp in immunological responses, in carcinogenesis, and in clinical outcome requires further investigation both in HPV-negative and HPV-positive OPSCC.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

JH, TS, CH, TA, AM, LJ, HM, and AKK designed the study; AKK, HM, LJ, HKM, RR-B and TA acquired and analyzed the data; all Authors participated in interpretation of the results, drafting, and revision of the manuscript, and in the decision to submit.

Acknowledgements

We thank Pia Saarinen for technical assistance.

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Received August 15, 2022
Revised September 6, 2022
Accepted September 8, 2022