Analysis of Preoperative Serum Levels of MMP1, -2, and -9 in Patients with Site-specific Head and Neck Squamous Cell Cancer

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Abstract. *Background: Head and neck squamous cell cancer (HNSCC) includes tumors of various anatomical sites sharing common etiological factors. Serum levels of MMP1, MMP2, and MMP9 were analyzed in patients with oropharyngeal, laryngeal, and hypopharyngeal carcinomas in an effort to elucidate the pathobiology and in order to find useful biomarkers of site-specific HNSCC. Patients and Methods: The study group comprised of 46 patients with HNSCC (21 with oropharyngeal, 21 with laryngeal and 4 with hypopharyngeal cancer). Serum levels of MMP1, -2, and -9 were determined by the MAGPIX multiplex method. P16 protein was detected by immunohistochemistry. Serum levels of matrix metalloproteinases (MMPs) were correlated with clinicopathological features of carcinomas and were compared with respect to tumor site. Results: Significant correlations were confirmed between p16 positivity and oropharyngeal cancer, MMP1 and p16 positivity, and recurrence and smoking. Statistically significant differences in serum levels of MMPs between cancer of different locations were not found. Conclusion: MMP1 expression is significantly affected by smoking habit and by p16 and might mediate etiopathogenetical process in cancerogenesis of HNSCC. Our pilot study did not establish any utility of MMP1, -2, or -9 in clinical practice as diagnostic/prognostic markers.*

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Key Words: MMP1, MMP2, MMP9, serum tumor marker, head and neck squamous cell carcinoma, oropharyngeal, laryngeal, hypopharyngeal cancer.

In spite of the same histopathology, head and neck squamous cell carcinomas (HNSCC) are a heterogeneous group of tumors with different location, etiology and pathobiology, clinical behavior and prognosis (1). Well-known etiological factors of HNSCC are smoking habit, alcohol abuse and human papillomavirus (HPV) infection (2). Despite advancements in therapy of these tumors, prognosis has not shown any improvement, with many cases of recurrent disease. Moreover, widely differing clinical outcomes exist and thus the identification of useful prognostic and predictive biomarkers is required. A better understanding over the pathogenesis of HNSCC might help improve early detection, prognosis and therapeutics for these tumor types.

The matrix metalloproteinases (MMPs) are a large family of structurally and functionally related zinc-dependent proteinases that are capable of cleaving most extracellular matrix (ECM) components, as well as other biologically important proteins. MMPs play a crucial role in the spread of malignant tumors by modulation of local tumor cell invasion and distant metastasis, angiogenesis, and apoptosis (3). However, both tumor-promoting and -inhibitory effects of MMPs have been described. MMPs are expressed both in tumor cells and in neoplastic stromal cells, including predominantly fibroblasts and inflammatory cells (4). Therefore the biology of MMPs in cancer is very complex and requires the understanding of particular MMP pathways in specific tumor types in order to evaluate the diagnostic and prognostic function of MMPs and to develop targeted therapy for use in routine clinical practice. Tumors with similar histological features may have widely differing clinical outcomes and thus the identification of prognostic and predictive biomarkers may be valuable for determining appropriate clinical management strategies.

The aim of the present study was to analyze serum levels of MMP1, MMP2, and MMP9 in patients with

oropharyngeal, laryngeal, and hypopharyngeal HNSCC and assess the possible role of these different sites and their relationship to staging, grading and etiological factors of these tumors in the pathogenesis of HNSCC.

Patients and Methods

Patient population. The study group comprised of a total of 46 patients with HNSCC treated from July 2011 to December 2012 at the Department of Otorhinolaryngology and Head and Neck Surgery, Faculty Hospital in Hradec Kralove, Czech Republic. Selection criteria for patients included the initial histology-confirmed diagnosis of HNSCC and availability of clinicopathological data and follow-up information. All patients underwent surgery. HNSCCs were sub-grouped into oropharyngeal (21 patients), laryngeal (21 patients), and hypopharyngeal (four patients) carcinomas. Tumor staging was determined using TNM system of the International Union Against Cancer (5). Grading of tumors and p16 status were retrieved from pathological reports. Smokers and non-smokers were included in the study. Exclusion criteria of this study group were other malignancy, inflammatory disease and infection. All participants were informed about the research study and written informed consent was obtained from each patient and the study approved by the Ethics Committee, University Hospital Hradec Kralove (number 201105 S14P). Treatment decision-making was based on the clinical status of patients and on grading and staging of tumors. The clinicopathological profile of the study populations are shown in Table I.

Blood serum collection and procedures. Ten milliliters of peripheral blood were drawn from every patient before surgery from 7 to 9 a.m. using standardized phlebotomy procedures. Blood samples were collected without anticoagulant into red top vacutainers and allow to coagulate for 20 to 30 min at room temperature. Sera were separated by centrifugation and all specimens were immediately aliquoted, and stored in at −80˚C until analysis.

Multiplex serum analysis and sources of immunoassays. Human MMP1, MMP2, and MMP9 concentrations were determined using the new multiplex method MAGPIX (Luminex corp. Austin, TX, USA). MAGPIX combines a fluidics system, a mechanical system, an electronic system and an optical system with magnetic microspheres and complex computer analysis to perform multiplex assays. The sample moves through the fluid tubing to the optics module, transported by the drive fluid. In the optics module, a magnet holds the magnetic microspheres in place while first a red (classification) LED and then a green (reporter) LED illuminates them. They are imaged during each illumination using a CCD camera. After the images are recorded, the magnet withdraws, releasing the microspheres for transport to the waste fluid container and clearing the way for the next sample. xPONENT software analyzes the images, the red-illuminated images are used to classify the microspheres and the green-illuminated images to determine what elements of the sample have bonded to their surfaces. The software reports the results to the operator. Multiple bead-based immunoassays for human MMP (Panel 2, Milliplex MAP Magnetic Bead kit) were purchased from EMD Millipore Corporation, MA, USA.

Determination of p16 status. In our hospital (Faculty Hospital in Hradec Kralove, Czech Republic) $p16^{INK4A}$ (hereafter denoted as p16) expression is routinely evaluated by immunohistochemical method within the province of histological examination of every case of HNSCC. Therefore, p16 status data are available and were retrieved from hospital records for patients of the study group.

Briefly, immunohistochemistry was performed on tissue sections (4 μm thick) after deparaffinization and rehydration with the CINtec® Histology Kit (Roche mtm laboratories AG, Heidelberg, Germany). $p16^{INK4a}$ protein was detected with primary mouse monoclonal antibody (clone E6H4) and reagent product was visualized by dextran polymer conjugated with horseradish peroxidase and goat antimouse immunoglobulins and diaminobenzidine (DAB) as chromogen. Negative control slides were provided by the manufacturer. External positive tissue control was performed on the samples of grade III cervical intraepithelial neoplasia. Brown staining of tumor cell nuclei/cytoplasm was interpreted as a positive result. The p16 immunostaining was scored as follows: negative, 0-50% tumor cells stained; positive, 51-100% tumor cells stained.

Statistical analysis of data. All statistical analyses were performed with SAS 9.2 (Statistical Analysis Software release 9.2, SAS Institute Inc., Cary, North Carolina, USA). The association between p16 expression and clinicopathological features was examined using the Chi-square test or Fischer exact test as appropriate. The Wilcoxon two-sample test and Kruskal–Wallis test were used for estimation of statistical significance for association between serum levels of MMPs and clinicopathological features. *p*-Values less than 0.05 were considered to be significant.

Results

Clinicopathological data of the patients with HNSCC in the studied cohort are summarized in the Table I. Serum levels of MMP1, MMP2, and MMP9 were evaluated in all three sub-groups (oropharyngeal, laryngeal and hypopharyngeal) of HNSCC. The evaluation was focused on the correlation between serum levels of MMPs and the site of tumor, and the relationship between serum concentration of MMPs and clinicopathological features of HNSCC (tumor stage, grading, persistence/recurrence, lymph node status, p16 status, as well as sex, age and smoking habit of patients). Finally, we also evaluated correlation between p16 status and clinicopathological features of HNSCC.

With respect to serum levels of MMPs in relation to clinicopathological features of HNSCC, no significant correlations or differences between serum levels of the studied MMPs and the site of primary tumors were found (Tables II, III and IV). No correlations were also observed between serum levels of MMPs and traditional clinicopathological factors such as sex and age. There was a tendency only for MMP1 to be associated with the >60 years age group.

Taking all HNSCC tumors together (irrespective of tumor site), there were statistically significant differences in serum levels of MMP1 and MMP9 between low-stage and high-stage cancers ($p=0.0415$; $p=0.0439$, respectively) with higher serum values of both markers in high stage neoplasms. Moreover, statistically significant differences in

Table I. *The clinicopathological profile of the study population.*

Feature	N	Anatomic site					
		Oropharynx (n=21)		Hypopharynx (n=4)		Larynx (n=21)	
		N	%	N	%	N	%
Gender							
Male	38	17	81.0	3	75.0	18	85.7
Female	8	4	19.0	1	25.0	3	14.3
Age, years							
≤60	28	15	71.4	3	75.0	10	47.6
>60	18	6	28.6	1	25.0	11	52.4
Mean	60.00	58.24		58.50		62.05	
Median	60	57		60		61	
Min-max		46-72		53-61		46-85	
Smoking habit							
Non-smoker	14	12	57.1	0	0	2	9.5
Smoker	32	9	42.9	4	100.0	19	90.5
TNM staging[a]							
I-II	13	4	19.0	0	0	9	42.9
III-IV	33	17	81.0	4	100.0	12	57.1
Histological grading							
G1-2	32	15	71.4	2	50.0	6	28.6
G3	14	6	28.6	2	50.0	15	71.4
Nodal status							
N0	20	5	23.8	0	0	15	71.4
N1-3	26	16	76.2	4	100.0	6	28.6
p16 status							
+	17	17	81.0	0	0	0	0
–	29	4	19.0	4	100.0	21	100.0
p-Value				<0.0001[b]			
Local recurrence/ persistence[c]							
No	39	18	85.7	3	75.0	18	85.7
Yes	7	3	14.3	1	25.0	3	14.3

LQ: Lower quartile; UQ: upper quartile; SD: standard deviation. Difference is significant at $p<0.05$. [a]TNM Classification of Malignant Tumours, 7th edition (5); [b]Chi-square test (comparison of anatomic site); [c]follow-up: 12-30 months.

MMP1 serum concentrations were found between p16-negative and p16-positive cancers ($p=0.0179$), between non-recurrence/persistence and recurrence/persistence cancers ($p=0.0247$) and between smokers and non-smokers ($p=0.0447$) with higher serum values of MMP1 in p16-positive cancers, recurrence/persistence cancers and smokers. Significant differences in serum MMP2 levels were found between low-grade and high-grade tumors ($p=0.0114$), with higher MMP2 concentrations being found in less differentiated neoplasms. Concentrations of MMP1 were highest in high-grade tumors, although these differences were not significant. No significant correlations were proven between serum MMP concentrations and lymph node status. Higher serum MMP9 levels were found in high-grade HNSCC and in that with nodal involvement, although statistical significance was not reached.

For oropharyngeal cancer, there were significant differences in serum MMP2 levels between positive lymph node and negative lymph node status ($p=0.0481$) with higher MMP2 concentrations being found in positive lymph node status, as well as low-grade and high-grade tumors ($p=0.0140$), with higher MMP2 concentrations being found in less differentiated neoplasms (Figure 1). The other two studied MMPs did not have any significant correlation in this respect. No correlations were found between all three MMPs and other clinicopathological features (sex, age, stage, p16 status, smoking, recurrence/persistence).

In laryngeal cancer, significantly higher serum MMP1 levels were associated with smoking habit of patients ($p=0.0384$) (Figure 2). The only significant differences in serum MMP2 levels were found between low-grade and high-grade tumors ($p=0.0140$), with higher MMP2 concentrations

Table II. *Serum levels of MMP1.*

	Serum MMP1 (pg/l)						
	N	Mean	SD	LQ	UQ	Median	*p*-Value
Gender							
Male	38	10597.13	7170.86	4501.01	13451.61	9469.81	0.1755[a]
Female	8	6556.34	4036.37	4275.01	7943.50	5201.44	
Age, years							
≤60	28	9379.74	6569.45	4471.73	13139.95	7683.68	0.6791[a]
>60	18	10694.93	7437.28	4501.01	18146.11	9476.12	
Anatomic site							
Oropharynx	21	8163.82	6285.07	4467.54	9631.81	6081.31	0.1618[b]
Hypopharynx	4	14254.86	9410.08	8195.60	20314.12	12340.99	
Larynx	21	10794.38	6756.07	5287.14	15353.11	9644.44	
Smoking habit							
Non-smoker	14	7656.15	7464.36	3755.69	7712.99	4605.58	**0.0447[a]**
Smoker	32	10873.61	6474.26	5312.22	13374.72	9638.13	
TNM staging[c]							
I-II	13	6494.11	4267.75	3378.16	9307.80	5337.30	**0.0415[a]**
III-IV	33	11233.88	7281.09	5007.05	14033.54	9631.81	
Histological grading							
G1-2	32	8501.79	6037.52	4107.59	12125.04	7463.86	0.0551[a]
G3	14	13077.44	7798.57	5395.82	19270.05	10507.97	
Nodal status							
N0	20	8977.22	6303.49	4107.59	12125.04	8551.75	0.4486[a]
N1-3	26	10599.89	7320.73	4735.25	13451.61	7727.66	
p16 status							
+	17	6807.48	4972.94	3873.03	7712.99	4735.25	**0.0179[a]**
–	29	11703.94	7250.37	5337.30	15353.11	10255.51	
Local recurrence/persistence[d]							
No	39	9010.44	6675.61	4367.12	11574.83	7214.72	**0.0247[a]**
Yes	7	14819.21	6202.97	10952.43	19270.05	13297.83	

LQ: Lower quartile; UQ: upper quartile; SD: standard deviation. Differences in bold were significant at $p<0.05$. [a]Wilcoxon two-sample test; [b]Kruskal–Wallis test; [c]TNM Classification of Malignant Tumours, 7th edition (5); [d]follow-up: 12-30 months.

being found in less differentiated neoplasms (Figure 3). Serum MMP9 levels significantly differed according to response to treatment of laryngeal cancer ($p=0.0430$). Surprisingly, significantly lower serum MMP9 levels were found solely in recurrent/persistent laryngeal cancer (Figure 4).

No significant differences between studied types of MMPs and clinicopathological characteristics of hypopharyngeal cancer were found.

P16 positivity of HNSCC is considered to be associated with HPV etiology. However, other causes of p16 expression also are suggested. Statistically significant p16 positivity was proven in patients with HNSCC with lymph node metastases ($p=0.0366$), in non-smokers ($p=0.0001$) and in tumors without recurrence/persistence ($p=0.0278$). P16 positivity was statistically significantly associated with oropharyngeal cancer ($p<0.0001$) compared to hypopharyngeal and laryngeal tumors.

Discussion

MMPs are factors having multiple and sometimes opposing roles in cancer pathobiology (4, 6, 7). In addition to their enzymatic effects as degraders of ECM, MMPs have also non-enzymatic functions as regulators of signaling pathways, with subsequent impacts on cell survival, motility and spreading (8, 9). New functional aspects of MMP2 and MMP9 in activation of specific signaling pathways were recently described (10). Both functional effects and regulation of MMP expression seem to be MMP-type related (11). MMPs might be important potential target molecules for development of such drugs against tumors with increased MMP content. The increased levels of one or several types of MMPs have been documented in numerous tumors, namely lung, colorectal, breast and prostate carcinomas (4). However, the prognostic and predictive role of different

Table III. *Serum levels of MMP2*.

	Serum MMP2 (pg/l)						
	N	Mean	SD	LQ	UQ	Median	*p*-Value
Gender							
Male	38	66018.76	14742.84	56386.57	75551.75	66742.68	0.2956[a]
Female	8	72673.75	20898.77	60432.53	84434.78	76744.67	
Age, years							
≤60	28	64418.00	17777.33	54133.18	76160.47	63497.33	0.1413[a]
>60	18	71466.61	11668.82	63497.33	79167.76	70335.88	
Anatomic site							
Oropharynx	21	65751.90	19486.77	54893.39	76769.19	63952.73	0.8215[b]
Hypopharynx	4	67587.36	10227.50	59742.93	75431.80	66120.23	
Larynx	21	68522.07	13042.60	63497.33	75551.75	68830.64	
Smoking habit							
Non-smoker	14	73373.46	15099.53	63497.33	80349.75	72860.18	0.1248[a]
Smoker	32	64464.82	15714.30	54133.18	75347.45	63725.03	
TNM staging[c]							
I-II	13	62784.41	17287.65	56386.57	71398.77	68396.59	0.4533[a]
III-IV	33	68906.22	15264.47	57367.72	80349.75	68178.89	
Histological grading							
G1-2	32	63050.61	15647.46	53372.97	70974.11	63497.33	**0.0114[a]**
G3	14	76605.96	12441.61	69262.93	85726.13	75347.45	
Nodal status							
N0	20	63141.04	14784.47	54879.77	72860.18	65946.96	0.2051[a]
N1-3	26	70280.08	16325.13	58819.14	82681.12	68720.91	
p16 status							
+	17	66498.74	19459.82	54893.39	76769.19	63952.73	0.7343[a]
–	29	67573.25	13784.92	61187.47	75551.75	68396.59	
Local recurrence/persistence[d]							
No	39	66055.79	16448.58	54893.39	76364.76	68178.89	0.2766[a]
Yes	7	73418.16	11468.66	63497.33	85726.13	68396.59	

LQ: Lower quartile; UQ: upper quartile; SD: standard deviation. Differences in bold were significant at $p<0.05$. [a]Wilcoxon two-sample test; [b]Kruskal–Wallis test; [c]TNM Classification of Malignant Tumours, 7th edition (5); [d]follow-up: 12-30 months.

MMPs in miscellaneous cancer types remains controversial (4, 7, 12-17).

HNSCC embraces the broad scale of tumors from the oral cavity to the larynx having the same histological type. HNSCCs represent about 6% of all cancer cases worldwide, most of which are oropharyngeal and laryngeal (18). Generally HNSCC is considered to be an aggressive neoplasm with unfavorable prognosis, despite improvements of therapy in the past decades, and such carcinomas are often studied together as a single disease. However, the variety of behaviors of these tumors in different head and neck locations probably reflects miscellaneous pathways of cancerogenesis and specific intrinsic tumor properties which are known from clinical practice (19). The presence of locoregional lymph node metastases is an important prognostic factor of these types of cancer. Moreover, many cases of HNSCC are associated with occult neck node metastases, although the tumor may have been classified as having an N0 neck status (20). Therefore, the search for new prognostic biomarkers of HNSCC with reasonable clinical efficacy seems to be fully substantiated (21).

Although certain studies have focused on the diagnostic and prognostic role of MMPs in HNSCC, their results seem to be controversial and hardly comparable with each other as studies were performed on different cohorts of HNSCC by various methods and different types of MMPs were evaluated, namely MMP2, -3, -7, -8, -9, -13, -14,-15, and -16 (16, 22-27).

Little is currently known about serum levels of MMPs in site-specific head and neck cancer. Therefore, we focused our study on the most common subsets of HNSCC, oropharyngeal and laryngeal cancer, in an effort to make the pathogenesis of these tumors and the relationship of preoperative serum MMP1, MMP2, and MMP9 levels to clinicopathological features clear. Four hypopharyngeal carcinomas were also included in our study forming a separate sub-group. Hypopharyngeal cancer is a less frequent tumor type with poor prognosis and very common lymphatic metastasis (28,29). Hence, the separate

Table IV. *Serum levels of MMP9*.

	Serum MMP9 (pg/l)						
	N	Mean	SD	LQ	UQ	Median	*p*-Value
Gender							
Male	38	123921.24	63068.45	79844.27	156248.60	113974.83	0.2584[a]
Female	8	101903.16	54741.30	81034.45	106925.08	86434.62	
Age, years							
≤60	28	130881.05	66927.62	82320.53	156386.15	113714.69	0.2263[a]
>60	18	103309.05	49753.42	48825.75	144849.07	101799.34	
Anatomic site							
Oropharynx	21	132592.30	61335.77	95744.03	156523.69	112179.45	0.3031[b]
Hypopharynx	4	155605.97	99119.50	75360.35	235851.59	151686.94	
Larynx	21	100827.15	50257.62	57661.75	139591.13	91476.68	
Smoking habit							
Non-smoker	14	126294.13	65763.04	84887.18	164394.32	101451.82	0.8774[a]
Smoker	32	117378.58	60736.39	72487.29	153130.21	113062.48	
TNM staging[c]							
I-II	13	92941.49	56482.29	48825.75	115249.92	82739.23	**0.0439[a]**
III-IV	33	130787.66	61163.56	91476.68	156523.69	112699.73	
Histological grading							
G1-2	32	117132.90	63522.48	78181.93	151277.17	102734.37	0.4560[a]
G3	14	126855.68	59058.96	87982.06	156248.60	114398.98	
Nodal status							
N0	20	107422.90	59575.35	58383.07	147893.90	97927.79	0.2335[a]
N1-3	26	129837.47	62685.81	84887.18	156523.69	111527.25	
p16 status							
+	17	117933.53	54647.12	87982.06	151615.62	103798.03	0.9099[a]
–	29	121357.32	66398.65	67940.39	154644.79	115249.92	
Local recurrence/persistence[d]							
No	39	122396.34	58796.48	82739.23	154644.79	110875.04	0.3482[a]
Yes	7	107253.58	80233.61	47702.80	156248.60	67940.39	

LQ: Lower quartile; UQ: upper quartile; SD: standard deviation. Differences in bold were significant at $p<0.05$. [a]Wilcoxon two-sample test; [b]Kruskal–Wallis test; [c]TNM Classification of Malignant Tumours, 7th edition (5); [d]follow-up: 12-30 months.

Table V. *List of matrix metalloproteinases determined in the present study [adapted from Nelson et al. (30)]*.

MMP	Descriptive name	Principal substrates
MMP1	Interstitial collagenase	Fibrillar collagens I, II, III
MMP2	Gelatinase A	Collagens IV, V, elastin, fibronectin
MMP9 (matrilysin)	Gelatinase B	Collagen IV, V, elastin, gelatin I

study of various biomarkers in hypopharyngeal cancer seems to be important. Nodal involvement of all cases of hypopharyngeal cancer in our study corresponds with the reported higher potential for lymphatic spread of these tumors (29). Different pathogenesis in respect to different clinicopathological characteristics of oropharyngeal, laryngeal, and hypopharyngeal tumors are supposed.

For this reason, we aimed to evaluate particularly the prognostic preoperative potential and differences of MMP1, MMP2, and MMP9 levels in site-specific HNSCC. MMPs determined in this study and their characteristics are summarized in Table V (30). We intended to analyze and compare the MMP status in site-specific HNSCC, therefore we did not use a control group of healthy patients. Significant differences in serum MMP concentrations were documented by some authors between patients with HNSCC and control groups for MMP3, MMP9 and MMP13, but not for MMP2 (23, 24, 27). Similarly, Kawata *et al*. in their study did not find differences for MMP2 between patients with HNSCC and healthy individuals (31).

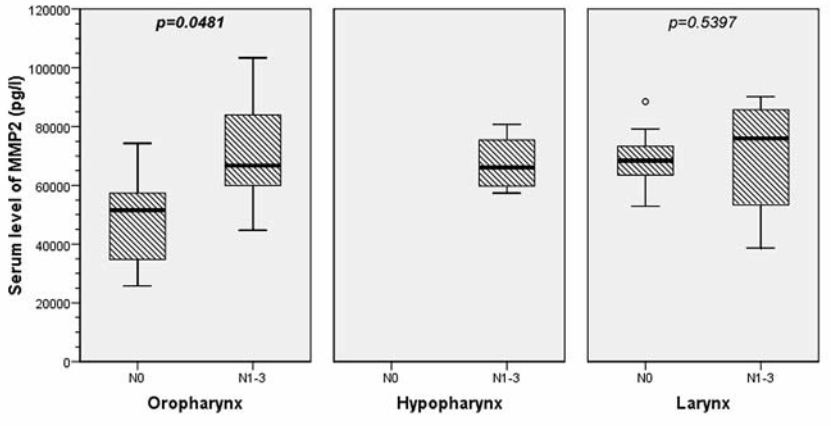

Figure 1. *Relationship of serum levels of MMP2 and nodal status (N0 versus N1-3) for different sites.*

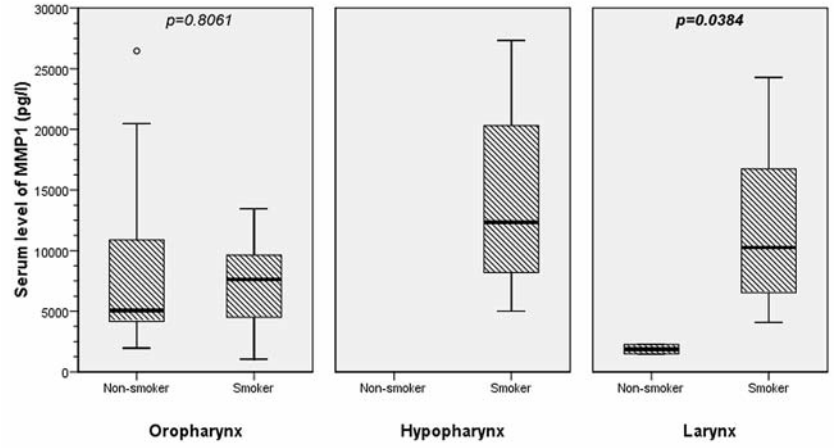

Figure 2. *Relationship of serum levels of MMP1 and cigarette smoking (non-smoker versus smoker) for different sites.*

With regard to serum levels of MMPs in relation to the anatomical site and clinicopathological features of HNSCC, our study did not find any statistically significant differences in serum levels of MMP1, MMP2, and MMP9 between oropharyngeal, hypopharyngeal, and laryngeal cancer. In spite of some HNSCC site-specific differences in serum MMP1 and MMP9 concentrations (MMP1 levels were the highest in hypopharyngeal and the lowest in oropharyngeal tumors; and MMP9 was the highest in hypopharyngeal and the lowest in laryngeal tumors), statistical significance was not reached (Table III and IV). For this reason, no role of MMPs is expected in the pathobiology of individual cancer groups. The potential role of MMPs in progression of hypopharyngeal HNSCC should be verified in larger cohort.

Taking all head and neck cancer together, in our study, no correlation was found between serum levels of any of the three MMPs and the traditional clinicopathological factors sex, age, tumor grading and lymph node status. Similarly, Kawata *et al*. described no correlation between serum levels of MMP2 and MMP9 and lymph node metastases, although cancer tissue concentration of MMP2 was significantly higher in patients with lymph node metastases (31). As opposed to significant correlations revealed by us among serum levels of MMP1 and MMP9 and stage of HNSCC, the study of Ruokolainen *et al*. found no association between serum MMP9 levels and tumor stage and other clinicopathological data. Nor were there any differences in preoperative serum MMP levels between tumor types, nor prognostic significance found (32). Moreover, the same authors did not confirm in their further study a prognostic effect of preoperative serum MMP2 level, although the prognostic role of tumor tissue MMP2 immunoreactivity was significant. Why MMPs derived from tumor are not reflected in the serum remains unclear (33).

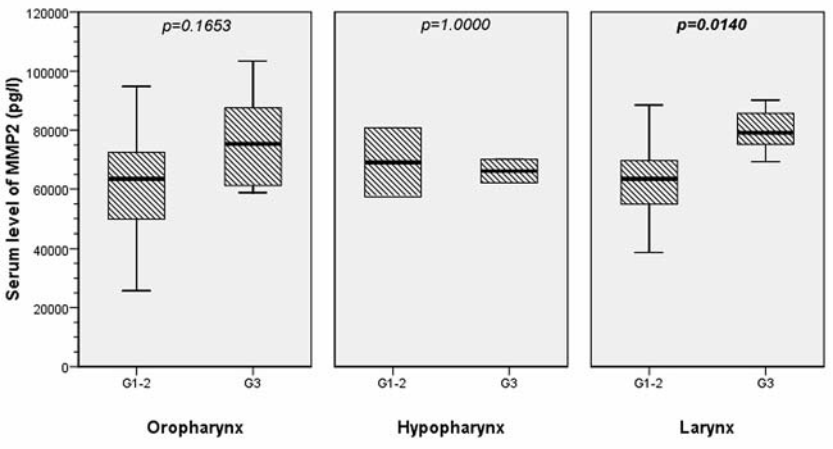

Figure 3. *Relationship of serum levels of MMP2 and histological grading (G1-2 versus G3) for different sites.*

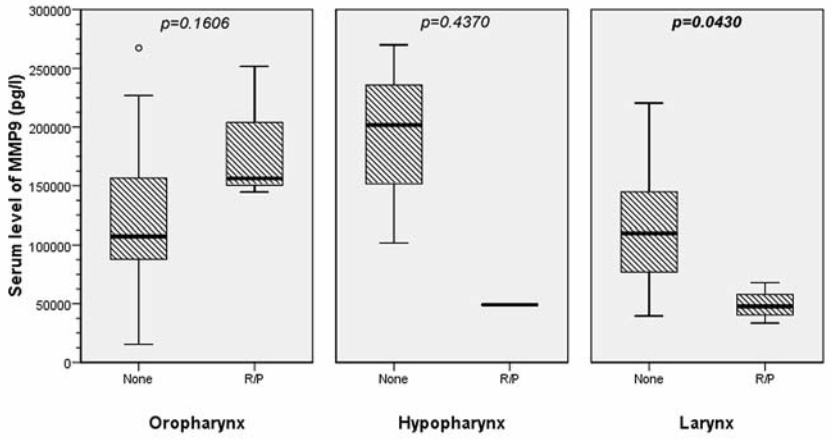

Figure 4. *Relationship of serum levels of MMP9 and tumor recurrence/persistence (R/P) (none versus R/P) for different sites.*

Within the boundaries of every studied cancer group, significant correlations were found only between serum MMP2 level and lymph node status in oropharyngeal cancer and between serum MMP2 level and tumor grade in both oropharyngeal and laryngeal carcinomas. Increased serum MMP9 level correlated with recurrence/persistence of laryngeal cancer, while in oropharyngeal cancer we found no relationship between MMP9 and stage of tumors. Immunohistochemical correlation between high MMP9 expression and high T and N stage of oropharyngeal cancer was reported by Dunne *et al*. (34). Gou *et al*. came to a similar conclusion and described a significant relationship between immunohistochemical expression of MMP2 and MMP9 and poor prognosis of laryngeal cancer (35). These results may indicate the partial role of MMP1, MMP2 and MMP9 in local spread of HNSCC without cancer site-specific predilection.

Cigarette smoking and alcohol abuse are major risk factors for developing HNSCC, predominantly laryngeal carcinoma. In our study, we found that 100% of patients with hypopharyngeal and 90.5% of those with laryngeal HNSCC were smokers. Cigarette smoke is thought to cause up-regulation of MMPs. Particularly circulating MMP9 is elevated by smoking-induced neutrophil and macrophage expression in the inflammatory state (36, 37). Correlation of higher serum MMP1 levels with smoking in patients with laryngeal cancer in our study is in accordance with previously reported enhanced expression of MMP1 due to induction of MMP1 by cigarette smoke in lung epithelial cells (38). This induction of the *MMP1* gene by cigarette smoke may be also influenced by polymorphisms of *MMP1* promoter (39). As correlation between elevated serum levels of MMPs and smoking was not confirmed in oropharyngeal

and hypopharyngeal cancer in our study, it seems probable that MMP1 might represent the most important regulator of cancer pathway in laryngeal cancer in smokers.

HPV infection is another etiological factor associated with HNSCC, namely with oropharyngeal cancer (40). Some authors described the influence of HPV on the overexpression of MMP2 and MMP9 (41, 42). P16 protein seems to be a surrogate marker for HPV-induced oncogenesis, being up-regulated in HPV-positive cases (2, 43, 44). p16 is suggested to be the most significant prognostic factor not only in oropharyngeal, but also in laryngeal cancer (19, 45, 46). Moreover, p16 overexpression has been documented to be associated with better response to therapy and favorable clinical outcome in patients with laryngeal/oropharyngeal squamous cell carcinomas despite a lack of detectable HPV DNA in some cases (47, 48). However, p16 overexpression did not correlate to HPV positivity and survival in hypopharyngeal cancer (49). Wang *et al*. described the inhibitory effect of p16 on MMP2 expression in human lung cancer cells (50). This fact may play key role in clinical behavior of p16-positive tumors. We found in our study significant correlation between low serum MMP1 concentration and p16 positivity in the whole cohort of HNSCC, but not in distinct cancer groups. The significant correlation found between p16 positivity and lymph node metastases in patients with HNSCC in our study does not go against the well-known favorable clinical outcome in patients with p16-positive HNSCC because the presence of lymph node metastases in oropharyngeal cancer with overexpression of p16 are not considered a negative prognostic factor (51).

Various investigative techniques have been used to study individual MMPs in different types of malignant tumors, namely immunohistochemistry, *in situ* zymography, reverse transcriptase-polymerase chain reaction, enzyme immunoassay and multiplex analysis (xMAP technology) (3, 52-54). The importance of immunohistochemical techniques lies in their applicability to formalin-fixed, wax-embedded tissue samples, facilitating exact antigen location within a tumor, as well as use of archival material in retrospective studies (3, 52). This method evaluates MMP expression of both active and inactive protein. Immunohistochemistry reflects the site of production of proteins, but requires cancer tissue samples. On the contrary, serum analysis may be performed before or during treatment. xMAP technology has been added to the innovative techniques of MMP assessment (54, 55). MAGPIX is the novel modification of this multiplex technology. This affordable system can perform up to 50 tests in a single reaction volume, greatly reducing sample input, reagents and labor while improving productivity. We have used this novel multianalyte technology allowing simultaneous measurement of multiple serum biomarkers.

In conclusion, MMP1, MMP2 and MMP9 can be considered as potential factors regulating pathogenesis, progression and spread of HNSCC. None of the studied MMPs seems to play a fundamental role in the pathobiology of oropharyngeal, laryngeal, and hypopharyngeal cancer. Because statistical significance between serum levels of MMP1, MMP2, and MMP9 and clinicopathological features of site-specific HNSCC is variable, blood concentrations of investigated MMPs cannot serve in clinical practice as prognostic tumor markers of these cancer types. A strongly significant link between p16 positivity and oropharyngeal cancer was, however, confirmed in accordance with previous studies. Serum MMP1 levels were significantly influenced by smoking and p16 expression, pointing to the potential role of this enzyme in the etiopathogenesis of HNSCC. Further research is required to determine pathways of cancerogenesis of oropharyngeal, laryngeal, and hypopharyngeal cancer, and to establish the exact role of all MMPs in pathobiology of site-specific HNSCC.

Acknowledgements

This study was supported by research projects of Charles University in Prague, Czech Republic GAUK No. 444311 and by the Charles University Research Fund (project number P36), by research project of Ministry of Health (Czech Republic) No. 00669806 (Faculty Hospital Plzen).

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Received July 24, 2014
Revised September 8, 2014
Accepted September 15, 2014