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Serum matrix metalloproteinase-2 in head and neck squamous cell carcinoma is associated with tumor differentiation

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Abstract

Matrix metalloproteinases (MMPs) play an important role in cancer invasion and metastasis. Elevated expression of MMPs has previously been found in a wide variety of cancers including head and neck squamous cell carcinomas (HNSCC). The purpose of this study was to assess the level of MMP-2 in the sera of HNSCC patients in relation to clinicopathological features of cancer. Serum samples from sixty-five patients (56M/9F, 59.11±9.02 years) with HNSCC were evaluated by ELISA, before and 48 hours after surgery. Forty healthy volunteers served as controls (29M/11F, 56.21±12.13 years). Serum MMP-2 concentration in relation to the degree of tumor differentiation in HNSCC was found to be statistically significant both before (P = 0.012) and after surgery (P = 0.041). A slightly decrease of MMP-2 in sera of patients with high tumor stage, tumor size and positive lymph nodes has been detected after surgery as compared to the concentrations measured before. No statistically significant association was found between serum MMP-2 level and tumor stage, tumor size, and lymph node involvement. Our data suggest that MMP-2 might be used as a marker of tumor differentiation and as well as a significant predictor of HNSCC progression.

Keywords: head and neck squamous cell carcinoma, matrix metalloproteinase-2, serum sample, histological tumor grade, marker of tumor differentiation

1. Introduction

Head and neck cancer (HNC) is the sixth most common cancer worldwide (PARKIN & al. [1]) and has an increasing incidence rate, with poor prognosis. HNC includes the following subsites: oral cavity, hypopharynx, larynx, nasopharynx, oropharynx, paranasal sinuses, nasal cavity and salivary glands. Over 90% are squamous cell carcinomas (head and neck squamous cell carcinoma - HNSCC), arising from the epithelial cells that line the mucosal surfaces of the head and neck (SUH & al. [2]). HNSCC is the most invalidating and visible disease, afflicting vital functions of human body as voice, breathing and swallowing. Spontaneous evolution of HNSCC is long time asymptomatic and the survival rate is about 50%, despite the advanced oncological treatments. Five-year survival was higher in women

(51%) than men (39%) (GREGOIR & al. [3]). Prognosis and treatment depend on the tumor stage (KOZIOROWSKI & al. [4]; STANCIU & al. [5]). Airways and digestive endoscopies are the current screening methods for detecting HNSCC. Unfortunately, the early diagnosis, predictions of tumor infiltration and metastasis, and prognosis based on clinical parameters are still difficult because of lacking in other diagnostic methods. Finding of reliable tumor markers could improve the management of the disease allowing more efficient treatments and better long-time survival. Clinical studies reveal that matrix metalloproteinases (MMPs) are secreted during the growth, invasion, metastasis, and angiogenesis of tumors, and affect the surrounding microenvironment, causing dynamic changes (KESSENBROCK & al. [6]; NAGASE & al. [7]). An important issue in HNSCC infiltration and metastasis is the degradation of the basement membrane (BM) between the epithelium and lamina propria, around cancer clusters, and surrounding vascular structures (GARAMSZEGI & al. [8]; EGEBLAD & al. [9]). High levels of proteases facilitate degradation of BM and extracellular matrix (ECM), and allow tumor cells to migrate and metastasize the vascular and lymphatic systems (FAN & al. [10]; STANCIU & al. [11]). MMP-2 (gelatinase A) is able to degrade connective tissue, and type-IV collagen, which is a major component of BM, mediating in this way invasion and metastasis of HNSCC. The association of the expression of MMP-2 with tumor invasion and nodal involvement has previously been found in squamous cell carcinoma, and its utility has been proven in oral cancers (FAN & al. [10]; [PATEL & al. [12]; PATEL & al. [13]; SINGH & al. [14]; MONTEIRO-AMADO & al. [15]). In our present study, we aimed to investigate the level of MMP-2 in the sera of HNSCC patients before and 48 hours after surgical treatment and its relationship with the tumor stage, size, histological grade and lymph node involvement in order to determine if these results can be used to assess the prognosis in HNSCC patients.

2. Materials and Methods

2.1. Patients and study protocol: The study included 65 patients with HNSCC (56M/9F) admitted to the Head & Neck Surgery Clinic of Coltea Clinical Hospital during 2014-2015. The diagnostic workup of HNSCC included history and physical examination, additional imaging (chest Radiograph, barium swallow, high resolution contrast-enhanced MRI or CT scan of head and neck or PET scan with ^{18}F-FDG, for an efficient metastatic workup), complete blood tests and endoscopic-guided biopsy (laryngoscopy, bronchoscopy, esophagoscopy – for assessing tumoral stages as well as detecting the possible existence of a second primary cancer). The mean age of the patients was 59.11±9.02 years (range, 37-80 years). The cancer stage, tumor size, and lymph node involvement were evaluated according to AJCC (American Joint Cancer Committee on Cancer) [16]. The histological grading of the tumors was reviewed and classified according to WHO Classification of Head and Neck Tumors. All the patients were investigated before and 48 hours after surgical treatment. They have been treated by surgical resection of the primary tumor associated with neck dissection for lymph node involvement. The oncologic surgical techniques that were used took into account the local invasion and tumoral stage, opting for either radical neck dissection (removal of all five lymph node groups along with the internal jugular vein, sternocleidomastoid muscle and spinal accessory nerve), modified radical neck dissection (removal of all five lymph nodes groups and one or two of other three structures mentioned at radical dissection) or selective neck dissection (removal of only those lymph nodes that present the highest risk for metastasis). A number of 40 healthy volunteers (29M/11F) were randomly selected. The mean age of the healthy controls was 56.21±12.13 years (range, 32-74 years). The study

was approved by the Ethical Committee of the Coltea Clinical Hospital. Enrolled patients and volunteers signed an informed consent for the use of their samples.

2.2. Biomarker measurements: In all patients and volunteers blood specimens were collected by venous puncture before and 2 days after surgical treatment. The venous blood was harvested in serum separator Vacutainer test tubes with gel and processed in maximum thirty minutes, because MMP-2 degrades in a very short time. Centrifugation was done at 4°C for 15 minutes at 3000 rpm. Serum samples were then transferred into labeled cryo-vials and frozen at -70°C. MMP-2 levels were detected using ELISA (Enzyme-Linked Immunosorbent Assay) technique. The kit for the quantitative determination of human MMP-2 was purchased from R&D Systems Inc. (Minneapolis, USA). Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision and in forty separate assays to assess inter-assay precision. CVs for MMP-2 were in a range between 5 and 6.9%. The assay was performed in duplicate according to the manufacturers' recommendations and in such a way minimize any effects of repeated freeze-thaw cycles.

2.3. Statistical analysis: All statistical analyses were performed using SPSS software system (SPSS for Windows, version 7.0). The analysis of the tumor stage, tumor size, nodal involvement, histological grade was separately done, according to the MMP-2 serum concentrations. The serum levels of MMP-2 did not follow a normal distribution based on Kolmogorov–Smirnov test. Therefore, nonparametric statistical analyses have been used. The Wilcoxon signed-rank test was used to compare different groups and subgroups (HNSCC *vs.* healthy controls; G1+G2 *vs*. G3; I+II vs. III+IV; T1+T2 *vs.* T3+T4 or N0 *vs.* N1 + N2 + N3). The statistical significance was determined with the X2 test. Differences were considered significant if P-value was <0.05.

3. Results and Discussions

3.1. Results

Clinical and histopathological data were collected by patients' medical records and there are presented in Table 1. Tumor grading showed that 63.1% of the patients had well-differentiated tumors (G1), 20% moderately-differentiated (G2), and 16.9% poorly-differentiated tumors (G3). According to staging (TNM), 20% of the patients were classified as stage I, 15.4% as stage II, 16.9% as stage III and 47.7% as stage IV (Table 1). Of the 65 HNSCC patients, 39 were pT_{4a}. In most cases it has been observed the presence of lymph node metastases that coexist with the multifocal form of HNSCC.

Table 1. Patient characteristics

Characteristic	Number	%
	N=65	
Age (yr)[a]	59.11± 9.02	-
Gender		
Male	56	86.2
Female	9	13.8
Tumor site		
Pharyngo-larynx	19	29.2
Base of tongue	16	24.6
Tonsil	12	18.5
Piriform sinus	10	15.4
Larynx	4	6.2
Tongue	3	4.6
Lip	1	1.5

Histological tumor grade		
G1	41	63.1
G2	13	20.0
G3	11	16.9
Tumor stage		
I	13	20.0
II	10	15.4
III	11	16.9
IV	31	47.7
Tumor size		
T1	12	18.5
T2	6	9.2
T3	8	12.3
T4	39	60
Lymph node involvement		
No (N0)	31	47.7
Yes (N1+N2+N3)	34	52.3
[a] Mean±SD		

As can be seen in Figure 1, comparing the HNSCC group with healthy control group, there were no significant differences in the serum MMP-2 concentration (150.0 ng/mL, IQR: 129.8-193.4 ng/mL *vs.* 160.1 ng/mL, IQR: 127.2-189.3, P = 0.321). However, we noticed that serum levels of MMP-2 were slightly lower in HNSCC patients than in healthy controls. More than that, the median MMP-2 levels calculated before surgery had almost the same value as those measured 48 hours after surgery (150.0 ng/mL, IQR: 129.8-193.4 ng/mL *vs*. 145.4 ng/mL, IQR: 126.7-190.6 ng/mL, P = 0.671).

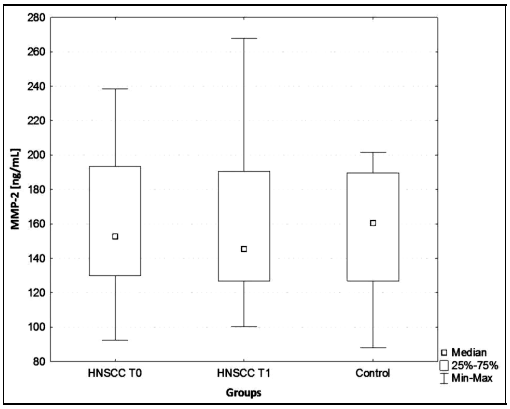

Figure 1. MMP-2 levels in the group of patients with HNSCC (before surgery - T0 and 48 hours after surgery - T1) and healthy control group. Lines denote median, boxes represent interquartile ranges and whiskers total ranges.

The tumor stages I and II were analyzed as one group (stage I+II), whereas stages III and IV as stage III+IV, because of small numbers of patients in the particular subgroups. Similarly, the T1 and T2 patients were analyzed as T1+T2 subgroup, whereas T3 and T4 as T3+T4 subgroup; G1 and G2 patients were analyzed as G1+G2 subgroup, whereas G3 remained as a single G3 subgroup; N1, N2, and N3 patients as N1 + N2 + N3 subgroup, whereas N0 remained as a single N0 subgroup (Table 2).

Concentrations (median and IQR) of MMP-2 in the sera of HNSCC patients before and 48 hours after surgery are presented in Table 2.

Statistically significant differences in serum levels of MMP-2 were observed between G1+G2 and G3 subgroups, related to the degree of differentiation of the tumor cells. As shown in Table 2, MMP-2 concentrations were higher in patients with poor tumor differentiation (G3) compared to those measured in patients with well-differentiated tumors and moderate tumor differentiation (G1+G2), both before ($P = 0.012$) and after surgery ($P = 0.041$), and in healthy control group ($P = 0.037$). We didn't find any statistically significant difference in the serum MMP-2 concentration between I+II and III+IV, T1+T2 and T3+T4, N0 and N1+N2+N3 subgroups. As can be seen from Table 2, MMP-2 levels were lower in the sera of HNSCC subjects with lymphatic nodes involved (N1+N2+N3 subgroup) than in patients without nodal metastases (N0 subgroup), both before and after surgery. Also, it has been observed that after surgery, the concentrations of MMP-2 were lower in patients with stage III+IV than in I+II subgroup and in T3+T4 tumors than in T1+T2. All the differences did not reach a statistical significance.

Table 2. Relationship between MMP-2 expression and clinicopathological features in HNSCC patients before and 48 hours after surgery

Variable	MMP-2, ng/mL (IQR)		
	Before surgery	**After surgery**	**P value**
Histological tumor grade			
G1+G2	135.1 (133.1 - 154.4)	138.1 (136.3 - 165.2)	0.572
G3	197.8 (134.9 - 220.5)	198.1 (145.5 - 238.5)	0.663
P value between G1+G2 and G3	0.012	0.041	-
Tumor stage			
I+II	152.7 (130.2-170.6)	148.2 (128.6-152.3)	0.445
III+IV	157.2 (135.9-180.5)	133.8 (122.3-151.6)	0.062
P value between I+II and III+IV	0.467	0.668	-
Tumor size			
T1+T2	148.5 (130.2-170.6)	148.2 (127.2-198.4)	0.872
T3+T4	157.5 (138.6-181.3)	131.8 (121.2-152.4)	0.059
P value between T1+T2 and T3+T4	0.468	0.668	-
Lymph node involvement			
N0	155.0 (140.5-176.8)	154.8 (138.8-183.5)	0.835
N1+N2+N3	153.1 (128.0-207.0)	122.2 (118.4-173.7)	0.054
P value betweenN0 and N1+N2+N3	0.852	0.782	-

3.2. Discussions

The role of tumor markers in the early-stage cancer, regardless of its location, is still a subject of debate. There are types of cancer, in which the level of a specific tumor marker in the blood is taken into account in assigning a stage ([STANCIU & al. [17]). Our interest was in studying of MMPs, that represent the potential for invasion and metastasis in HNSCC. Most of the papers have commonly identified only one or two MMPs, usually MMP-2 and/or MMP-9, associated with lymph node metastasis and poor outcome in a given head and neck site ([FAN & al. [10]; PATEL & al. [12]; PATEL & al. [13]; SINGH & al. [14]; MONTEIRO-AMADO & al. [15]; BURDUK & al. [18]; ROSENTHAL & al. [19]; XIE & al. [20]; KATAYAMA & al. [21]; RIEDEL & al. [22], LI & al. [23]). In almost all of the studies found in the literature, the authors analyzed MMP-2 mRNA or immunohistochemical

reactivity, emphasizing that increased active MMP-2 has prognostic importance in laryngeal and oral cavity squamous cell carcinoma ([PATEL & al. [12]; SINGH & al. [14]; MONTEIRO-AMADO & al. [15]; BURDUK & al. [18]; ROSENTHAL & al. [19]). Unfortunately, MMP-2 mRNA or immunohistochemical reactivity may not correspond to *in vivo* enzymatic activity of MMP-2. This why we decided to investigate the level of MMP-2 in the sera of HNSCC patients in relation to clinicopathological features of cancer. Current study revealed that the serum levels of MMP-2 were slightly lower in HNSCC patients than in healthy subjects. Our findings are in line with the results obtained by other authors (RIEDEL & al. [22], WAAS & al. [24]; GROBLEWSKA & al. [25]), who have reported that no significant difference of plasma pro-MMP-2 and serum MMP-2 levels was seen when comparing patients with squamous cell carcinoma and normal controls. Unlike our findings, LOTFI & al. [26] reported a significant increase in serum levels of MMP-2 in patients with oral squamous cell carcinoma compared to control subjects, in accordance with previous studies which have not measured serum levels of MMPs, but have also shown significant differences between expression of MMP-2 compared to normal tissues and normal subjects (PATEL & al. [12]; PATEL & al. [13]; SINGH & al. [14]). Moreover, we expected to see a statistically significant difference between serum levels of MMP-2 measured before and after tumor/lymph nodes removal, but the results did not confirm our assumption. Actually, the serum concentrations measured before and after surgery were broadly similar to those measured in healthy subjects. Although there was no significant difference in the level of MMP-2, however a slightly decrease has been detected 48 hours after surgery as compared to the concentrations measured before. Thus, we observed that the reduction of MMP-2 concentration was higher in patients with stage III+IV than in I+II (14.9% *vs*. 2.9%, P = 0.062), T3+T4 tumors than in T1+T2 (16.3% *vs*. 0.2%, P = 0.059), and in patients with lymphatic node involved N1+N2+N3 than in N0 subgroup (20.2% *vs.* 0.13%, P=0.054). Decreased levels of MMP-2 in sera of patients with high tumor stage, tumor size and positive lymph nodes was due to tumor/lymph nodes removal and shows the involvement of MMP-2 in the tumor growth and metastasis. Several studies evaluating HNSCC have reported different results regarding the role of MMP-2 expression in the cancer diagnosis and progression. The most important finding of our study was that the concentrations of MMP-2 in the sera of patients with HNSCC were related to the degree of differentiation of the tumor cells both before (P = 0.012) and 48 hours after surgery (P = 0.041). This result was in accordance with the study of FAN & al. [10] who had showed that expression of MMP-2 was differentiated among tumors. FAN & al. [10] and MONTEIRO-AMADO & al. [15] reported an independent prognostic significance for histological grade, including even small size tumors and providing evidence for the importance of routine assessment of histological grade in HNSCC in addition to tumor stage, tumor size, and lymph node involvement. When analyzing the relationship between MMP-2 and tumor stage, tumor size, and nodal involvement, the present study did not show any statistical significance. Nevertheless, there has been a trend toward decreased MMP-2 serum concentrations in more advanced tumors, more exactly in subjects with positive lymph nodes (N1+N2+N3 subgroup) than in patients with negative lymph nodes (N0 subgroup). The decrease of serum MMP-2 levels in subjects with positive lymph nodes might be caused by the formation of MMP–TIMP complexes in HNSCC progression. This trend is opposite to the results of LI & al. [23] and LOTFI & al. [26] who showed that higher serum levels of MMP-2 were significantly correlated with nodal involvement and mode of tumor invasion. However, our findings are in accordance with the studies of KATAYAMA & al. [21] and MAKINEN & al. [27], who have reported

no correlation between MMP-2 and tumor clinicopathologic findings including invasion and size of the tumor.

All differences between the results observed by cited authors and those obtained in present study could be due to differences in study design, study groups (smaller or larger, more or less homogeneous), variable stage or size of HNSCC, absence or presence of lymph nodes, various types of biological specimens study, as well as their evaluation methods. Although studies results were quite contrasting, they expressed in one way or another the role of MMP-2 in the evaluation of advanced or early stage of HNSCC.

4. Conclusion

Our data suggest that MMP-2 might be used as a marker of tumor differentiation and as well as a significant predictor of HNSCC progression. Further studies are needed to confirm these findings.

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