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Influence of Matrix Metalloproteinase 9 (MMP-9) on the Metastatic Behavior of Oropharyngeal Cancer

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Abstract. *Background: The role of the single matrix metalloproteinases (MMPs) in the metastatic process of squamous cell carcinomas (SCC) is still obscure. Materials and Methods: The MMP-9 expression was described immunohistochemically in 105 patients (40-79 years of age, mean: 57.84 years; 84 male, 21 female) suffering from oropharyngeal cancer (22x T1, 31x T2, 24x T3, 28x T4) with different neck stages (41x N0, 6x N1, 54x N2, 4x N3 neck). Results: A significant correlation between MMP-9 expression and T stage ($p<0.05$), N stage ($r=0.55$, $p<0.01$) and UICC stage ($r=0.55$, $p<0.01$) was revealed. Most remarkable was the high MMP-9 expression with simultaneously high UICC stages. Conclusion: The results give further indication that MMP-9 plays a role in the metastatic behavior of oropharyngeal SCC. It will be a project for the near future to create a standardized evaluation score of immuno-histological stainings to allow valid comparison of the results and published data.*

The expression and activation of proteolytic enzymes is essential for the invasiveness and metastatic spread of malignant tumors in order to induce tumor growth and destroy the basal membrane and extracellular matrix (1, 2). The prognosis of patients suffering from carcinomas of the upper aerodigestive tract is directly related to the presence or absence of lymphogenic metastasis (3, 4). With this background, it becomes clear why many study groups are working on the identification of risk factors to predict lymphogenic metastatic spread and aggressiveness of the local tumor growth. Meanwhile many histological and molecular biological factors have been proposed for their possible impact on the lymphogenic metastatic process.

In this context, special interest is being paid to a better knowledge of the matrix metalloproteinases (MMPs) (5), that are involved in the physiology of reconstruction and renewal processes of surrounding tissue. In particular, MMP-2 and -9, originating from the subfamily of gelatinases, seem to play important roles in the metastatic process of several carcinomas (6, 7), including not only SCC of the head and neck (8), but also malignant tumors of the lung (9), the prostate (10), the colon (11) and the bladder (12).

The aim of the present study was to investigate if the immunohistochemical detection of increased MMP-9 expression, which could easily be introduced into clinical practice, shows significant correlation to the aggressiveness and the metastatic behavior of malignomas of the oropharynx.

Materials and Methods

Materials. The analyzed oropharyngeal tumor specimens were taken from 105 patients (40-79 years of age, mean: 57.84 years; 84 male, 21 female) being treated at the Department of Otolaryngology, Head and Neck Surgery, of the Philipps University of Marburg, Germany, between June 1998 and December 2000. The T stage was classified for 22 patients as T1, for 31 patients as T2, for 24 patients as T3 and for 28 patients as T4 oropharyngeal cancer. A total of 41 patients had N0 necks, 6 patients had N1 necks, 54 patients had N2 necks and 4 patients even showed N3 metastasis. At the time of first diagnosis, 7 of the patients had pulmonary metastasis (M1). Tonsillar tissues were taken as reference specimens.

Methods. The avidin-biotin method was applied for detection of MMP-9 proteins. As preparation for the creation of the histological sections, the 105 paraffin blocks of the primaries were cut, paraffin was removed and then the sections were soaked in a decreasing alcohol series (100%-70%). In order to block endogenous peroxidase and thus false-positive disruptive reactions, the sections were rinsed with 200 ml methanol and 6 ml 30% H_2O_2 and distilled water. Normal serum (Normalserum, Sigma-Aldrich GmbH, 89502 Steinheim, Germany) was applied to the sections to avoid

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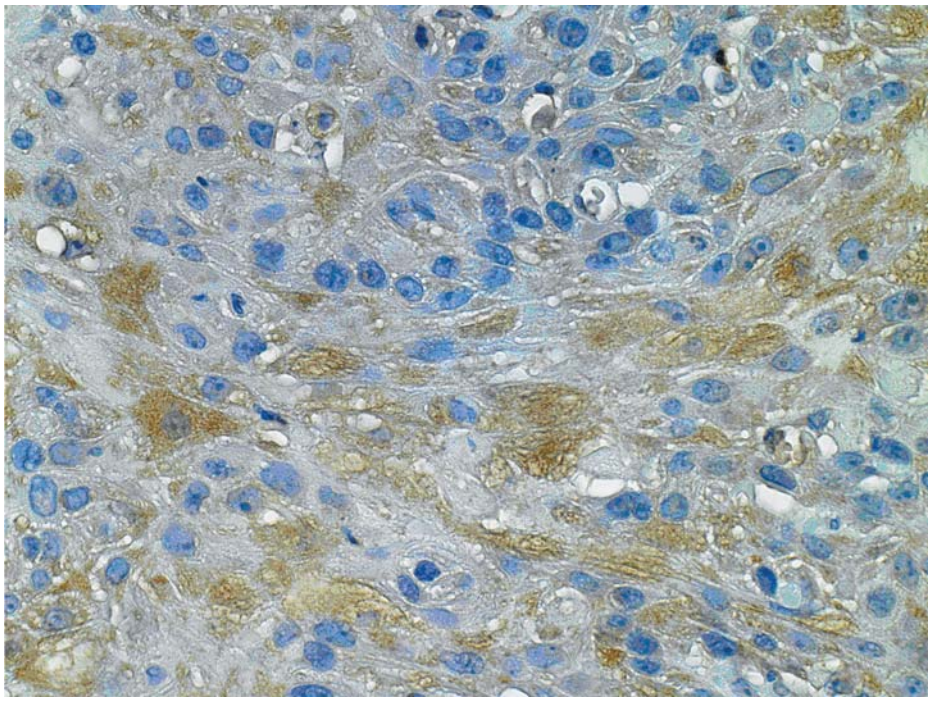

Figure 1. *Immunohistochemical staining of MMP-9 according to the ABC method of an oropharyngeal tumor that shows staining of less than 50% of the tumor cells or stroma tissue (40-fold enlargement) and thus can be classified into category 1+.*

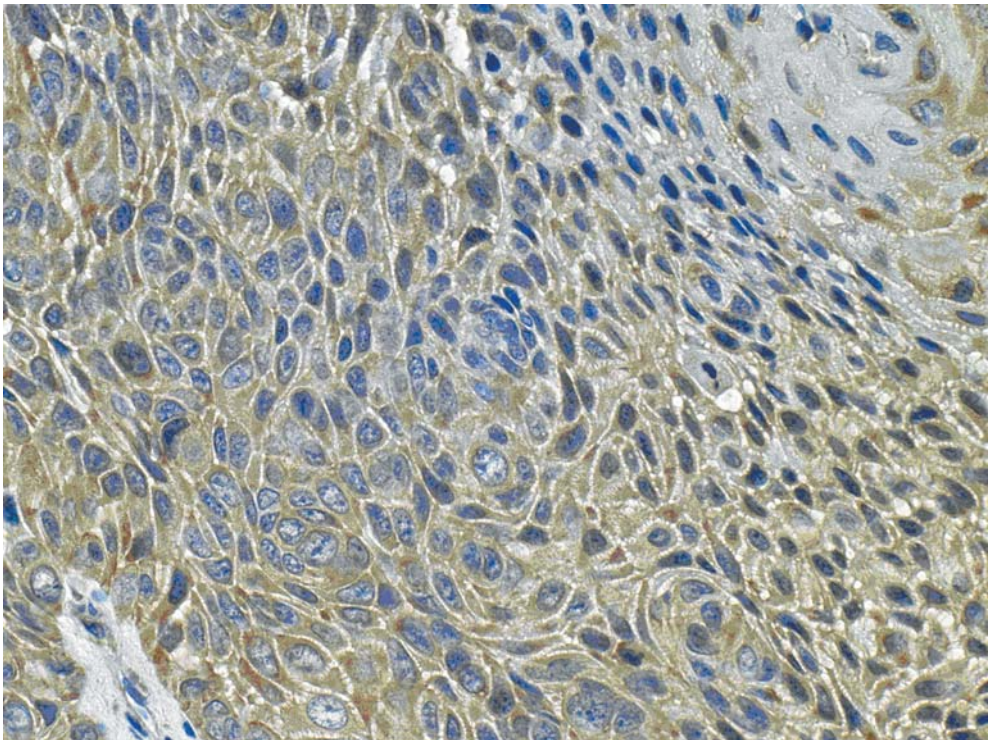

Figure 2. *Immunohistochemical staining of MMP-9 according to the ABC method of an oropharyngeal tumor that shows extended staining of tumor cells and stroma tissue (40-fold enlargement) and can thus be classified into category 3+.*

Table I. *Correlation between MMP-9 expression level and the T classification of oropharyngeal carcinomas.*

Expression level	T1	T2	T3	T4	Total
0	2	3	0	0	5
1+	8	5	6	5	24
2+	7	14	6	10	37
3+	5	9	12	13	39
Total	22	31	24	28	105

Table II. *Correlation between MMP-9 expression level and the involvement of cervical lymph nodes in oropharyngeal carcinomas.*

Expression level	Lymph node		Total
	negative (N0)	positive (N+)	
0	4	1	5
1+	20	4	24
2+	12	24	36
3+	5	35	40
Total	41/39%	64/61%	105

unspecific collagen reactions and reactions with endogenic IgGs. After removal of the normal serum from the section, the primary antibody (monoclonal anti-human MMP-9 antibody, clone 56-2A4, Oncogene Research Products, USA) was applied. After rinsing with PBS solution, the second antibody (polycloncal anti-mouse IgG, Dako A/S, Denmark) was applied. Finally the A-B complex was applied to the secondary antibody (Strept.-ABC-Komplex/HRP, Dako, Denmark). The counterstain of the section specimen was performed with haemalum (Hämalaun, Merck KG aA, 64271 Darmstadt, Germany) before the section specimens were soaked in an ascending alcohol series (50%-100%). Concentration of the section specimens was subsequently performed with corbit balsam (Corbit-Balsam, I. Hecht, Kiel-Hassee, Germany) for evaluation.

Statistical evaluation. The statistical evaluation of the collected data was performed with SPSS 11.0 for Windows, while a bivariant correlation test performed according to Spearman. The expression levels of MMP-9 were examined with regard to their correlation with clinical parameters such as age and gender of the patients, histological differentiation of the tumor, TNM classification, as well as the UICC stage.

Results

Evaluation system. The stainings (Figures 1, 2) were evaluated according to Kurahara *et al.* (13):

0 = nearly no staining of tumor cells/stroma
1+ = staining of less than 50% of the tumor cells and/or weak staining of stroma cells
2+ = staining of more than 50% of tumor cells and/or moderate staining of stroma cells
3+ = extended staining of tumor cells and/or high staining of stroma cells

Detection of MMP-9 signals in tissue. The immunohistochemical examination of the 105 oropharyngeal carcinomas showed MMP-9 expression in 95.2% (100/105) of the cases. The intensity of MMP-9 expression amounted to <50% of the tumor cells or the stroma tissue in 24 specimens, >50% of the tumor cells or stroma tissue in 37 cases, and an extensive tumor cell or stroma staining was revealed in 39 specimens. No simultaneously stained tonsillar epithelia showed any staining. No correlations between age or gender, or the histological grading and the detected MMP-9 expression level could be detected.

Correlation between MMP-9 expression and TNM classification/UICC stage or oropharyngeal carcinomas. The expression level for MMP-9 and T classification showed statistically significant ($p<0.05$) correlation (Table I). Further, the bivariant correlation test according to Pearson revealed a statistically relevant correlation ($r=0.55$, $p<0.01$) between the presence (N+ neck) and the absence (N0 neck) of cervical lymph node metastases (Table II). In this context, less aggressive oropharyngeal phenotypes without nodal metastasis showed low MMP-9 expression levels in 90.2% (37/41) of the cases. The aggressive phenotypes with cervical lymph node metastases, however, had MMP-9 expression of levels 2+ and 3+ in 98.4% (63/64) of the cases. Regarding the N stage, a significant correlation could also be detected ($r=0.59$, $p<0.01$), but it must be mentioned that the case number for stages N1 and N3 were relatively low (Table III). A statistically significant correlation ($r=0.55$, $p<0.01$) was found between MMP-9 expression and the international UICC tumor stage (Table IV). The high MMP-9 expression in high UICC stages is remarkable: 84.6% (22/26) were classified stages I/II and 98.7% of the UICC stages III/IV (78/79) had MMP-9 expression, while the expression pattern in stages III/IV corresponded to the higher MMP-9 expression levels of 2+ and 3+.

Discussion

Primary tumors with high metastatic potential are able to release several millions of cells into the circulation per day, while only several hundred have the ability to induce metastasis. Access to the venous vascular system is facilitated for the cell populations released by the primary by newly-

Table III. *Correlation between MMP-9 expression level and N stage of oropharyngeal carcinomas.*

Expression level	N1	N2	N3	Total
0	1	0	0	1
1+	1	3	0	4
2+	3	21	1	25
3+	1	30	3	34
Total	6	54	4	64

Table IV. *Correlation between MMP-9 expression level and UICC stage of oropharygneal carcinomas.*

Expression level	UICC I	UICC II	UICC I+II	UICC III	UICC IV	UICC III+IV	Total UICC I-IV
0	2	2	4	1	0	1	5
1+	7	5	12	7	5	12	24
2+	3	5	8	5	24	29	37
3+	1	1	2	2	35	37	39
Total	13	13	26	15	64	79	105

developed blood vessels having significant defects, which makes their barrier function ineffective (14). In this context, tumor angiogenesis must be mentioned; histopathological studies have demonstrated that high tumor vascularization is generally a sign of higher aggressiveness with early occurrence of distant metastases (15). Other investigations showed that not only VEGF (vascular endothelial growth factor) should be considered as a main angiogenic factor for tumor neoangiogenesis, but also MMPs, especially MMP-9 (16, 17). Additionally, MMP-9 was described in the context of squamous cell carcinomas of the head and neck and in non-parvicellular pulmonary cancer and its effect on a poor prognosis with increased neoangiogenesis factors such as VEGF was published (18, 19).

Metastatic spread also occurs *via* the lymph vessels. It must be assumed that lymphogenic metastatic spread occurs at the tumor margins, the so-called invasion front. From there, tumor cells may find their way to the regional lymph nodes as lymphangiosis carcinomatosa, and mature to micrometastasis. Micrometastases are defined as a metastatic carcinomatous focus <3 mm originating from a lymph node sinus with minimal alteration of the lymph node structure (20). Generally, this procedure may occur in over 300 different lymph nodes of the head and neck. Which lymph nodes will finally be affected depends on the density and the direction of the initial lymph vessels in the primary tumor area. Each tumor location has a preferred drainage into one or more lymph node groups; lymph node stations, however, may also be skipped. If the metastatic cell aggregation has not colonized the lymph nodes, or if the metastatic cell aggregation has become the focus of further metastases itself, invasion into the systemic venous drainage *via* lympho-hematogenous connections is possible.

For both metastatic routes, lysis of the basal membrane is the first step in a complicated metastatic process and represents the beginning of the invasion of a primary tumor that continues to the development of metastasis. Basal membranes limit most epithelial structures and represent a physiological barrier between histologically differentiated tissues. The lysis of this basal membrane and of components of the extracellular matrix is characteristic of malignant cells (21); in contrast, benign cells do not have this ability (22). The invasion process occurs in three steps, according to expert opinion: i) attachment of tumor cells to the cellular surface receptors of the matrix, such as integrin or cadherin, by means of glycoproteins, such as laminin or fibronectin; ii) local degradation of this matrix by cell-associated proteases, and, finally, iii) the tumor cell movement through the degraded matrix along chemotactic factors.

In this metastatic process, valid also for SCC of the head and neck, a group of proteinases plays a central role in the increased lytic activity. Beside the serine proteinases tissue and urokinase plasmino gene activator, those are the cysteine proteinases cathepsin B and L, the aspartate proteinases cathepsin D, and the so-called matrix metalloproteinases, which are most significant in this context (23). The expression of these MMPs is not decisive, but it is rather the correlation between them, or even the imbalance of these proteinases and their natural inhibitors (tissue inhibitors of metalloproteinases, TIMPs), that confers a higher lytic activity to the primary tumor.

It is assumed that MMP-9, as a significant representative of the gelatinases, plays an important role in the final degradation of fibrillar collagen. Epithelial cells, and especially keratinocytes, express gelatinase B (MMP-9), which is additionally stored in the secretory granula of neutrophils and eosinophils. Most investigations concluded that gelatinases are expressed in malignant tissue, some even describing production in the malignoma cells themselves (24). However, the majority of publications concentrated on an expression in the stroma (25).

Many studies confirmed that expression of MMP-9 occurred in macrophages, neutrophils, or fibroblasts found in the surroundings of tumor cells (26), while immunohistochemical examinations could show an expression of MMP-9 in tumor cell aggregations (27). Also in this context, the expression is concentrated on the invasive front of the malignoma, where not only the matrix is lysed,

but also growth and angiogenic factors that were previously bound to the extracellular matrix are released (28).

It would thus be of great scientific importance to integrate MMP-9 into a risk profile (29), in order to help integrate the metastatic potential of a primary tumor in the development of treatment strategies for SCC of the head and neck. Of main interest are primary tumors with clinically undetected cervical lymph node metastasis, *i.e.* an N0 stage.

With this background, the results of the present investigation correspond with the observations of other study groups (30, 31), that found a statistically relevant correlation of increased MMP-9 expression with the T stage, N stage and UICC stage. The specific role of gelatinase A (MMP-2) and gelatinase B (MMP-9) is documented for SCC of the oral cavity (32), and the increased expression of the gelatinases as a subfamily of the MMPs of the head and neck.

However, the current literature would not allow a statement regarding the prognostic relevance of MMP-9 expression for the lymphogenic metastatic behavior of carcinomas of the upper aerodigestive tract. This may be due to the lack of a standardized evaluation system of the expression patterns. In this context, each study group has its own system, which makes a valid assessment of the data very difficult. Furthermore, the intensity of MMP-9 signals only allows a quantitative statement on the expression of MMP-9 proteins and does not provide any information about the activity of MMP-9. Additionally, the simultaneous detection of TIMPs was not involved in the investigations.

The presented results give further indications that MMP-9 plays a role in the metastatic behavior of squamous cell carcinomas of the oropharynx. Due to the MMP-9 ability to destroy type IV collagen and other main components of the extracellular matrix and the basal membrane, a modulator function of MMP-9 for the malignant behavior of carcinomas of the head and neck can be assumed. An immediate aim must be the development of a standardized evaluation score of immunohistochemical staining. Only a standardized approach will allow for a valid assessment of the results and a comparison of published data at an international level.

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