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RT-PCR Expression Profiling of Matrix Metalloproteinases and their Specific Inhibitors in Cell Lines and Fresh Biopsies of Squamous Cell Carcinomas of the Head and Neck

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Abstract. *The expressions of MMP2, -7, -9, -13 and TIMP1, -2, -3 were examined in biopsies and cell lines of head and neck squamous cell carcinomas (HNSCC) to determine the association between the expression profile and TNM-staging of the primary. The expressions of MMP2, -7, -9, -13 and TIMP1, -2, -3 were analyzed in 30 HNSCC biopsies, 7 HNSCC cell lines and 1 keratinocyte cell line using RT-PCR. Negative correlation was determined between N-status and MMP13-RNA expression [Kendall-tau-b –0.404 (p=0.016), Spearman-rho –0.448 (p=0.014)], histological grading [Kendall-tau-b −0.291 (p=0.049), Spearman-rho −0,333 (p=0.048)], and MMP7 and TIMP2 expression [Kendall-tau-b −0.318 (p=0.045); Spearman-rho −0.353 (p=0.045)]. Positive correlation was determined between M-status and MMP9-RNA expression [Kendall-tau-b 0.341 (p=0.025), Spearman-rho 0.377 (p=0.024)] and MMP13 and TIMP2 expression [Kendall-tau-b 0.727 (p=0.037), Spearman-rho 0.850 (p=0.016)]. The results point to a role of the tested MMPs and TIMPs in the metastatic spread of HNSCC.*

In recent years, matrix metalloproteinases (MMPs) have been studied in two respects. On the one hand, whether new chemotherapeutic agents such as MMP inhibitors, affecting the proteolytic activity or synthesis of the protease, might have an effect on tumor progression was investigated (1-5). On the other hand, MMPs were evaluated as potential prognostic markers in tumor disease progression of many different tumor entities, including head and neck squamous cell carcinomas (HNSCC). Several groups found a correlation between MMP expression and prognosis such as in urothelial cancer (6), malignant melanoma (7), non-parvicellular lung cancer (8) and squamous cell carcinomas of the hypopharynx (5). Despite intensive research in this field, no final conclusion has been drawn regarding the significance of MMPs in malignancies in general and HNSCC specifically. Therefore, further studies are required to clarify this subject. The present study, using RT-PCR, investigated whether mRNA expression levels of MMPs -2, -7, -9 and -13 and their specific inhibitors TIMP1, -2 and -3 reveal a reproducible pattern specific for HNSCC cancer. Additionally, the aim of this study was to evaluate a possible correlation between expression patterns, tumor size, presence of lymph node metastases and histological grading, as well as location of the primary tumor.

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Materials and Methods

Materials. Thirty tumor tissue samples derived from 28 different patients (25 male, 3 female; 54.8±7.96 years of age) with HNSCC tumors were received during regularly scheduled tumor surgery, after informed consent of the patient. The tumor-associated data is summarized in Table I. Seven squamous cell carcinoma cell lines (UM-SCC-3, UM-SCC-27 and UT-SCC-16A, UT-SCC-16B, UT-SCC-19B, UT-SCC-24A and UT-SCC-24B) were kindly provided by T.E. Carey (University of Michigan, Ann Arbor, MI, USA) and R. Grénman (University of Turku, Turku, Finland), respectively (9). A keratinocyte cell line, derived from healthy oral mucosa, was used as a control.

Methods. Total RNA was isolated with the Qiagen RNeasy Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometry (LKB Ultrospec III, Pharmacia, Germany). cDNA was generated by reverse transcription using the "1st Strand cDNA Synthesis Kit for RT-PCR (AMV)" (Roche, Mannheim, Germany). PCR amplification was performed with the following settings: (i) 95˚C, 15 min; (ii) 95˚C, 1 min; (iii) 45˚C, 1 min; (iv) 72˚C, 3 min; (v) 35 cycles (ii)-(iv), (vi) 72˚C, 15 min. The MMP specific primers are indicated in Table II. Amplified PCR products were separated by electrophoresis (Kodak BioMax MP 015, New Haven, USA) on 2% agarose gels and stained with ethidium

Table I. *Clinicopathological parameters of patients who underwent excision of the tumor tissue specimen.*

No.	Gender	Age	T	N	M	G	Location
1	m	58	2	2	0	2	hypopharynx
2	m	64	2	2	1	3	larynx
3	m	57	2	0	0	2	oropharynx
4	m	64	2	0	0	3	hypopharynx
5	m	43	2	0	1	3	metastasis
6	m	61	3	3	0	2	metastasis
7	m	68	3	0	1	3	metastasis
8	m	53	3	0	0	2	oropharynx
9	m	44	3	2	0	2	hypopharynx
10	m	44	3	2	0	3	metastasis
11	m	55	3	2	0	3	hypopharynx
12	m	55	3	2	0	4	metastasis
13	m	56	3	2	0	3	larynx
14	m	61	3	2	0	2	oropharynx
15	m	48	3	2	0	2	hypopharynx
16	m	64	3	0	0	2	hypopharynx
17	m	45	3	2	0	3	hypopharynx
18	m	43	3	0	0	2	oropharynx
19	m	67	3	0	0	2	larynx
20	f	47	4	0	0	2	oropharynx
21	m	59	4	2	0	2	hypopharynx
22	m	68	4	2	0	2	oropharynx
23	f	55	4	2	x	3	oropharynx
24	m	57	4	2	0	1	oropharynx
25	m	51	4	2	0	2	hypopharynx
26	f	61	4	2	0	2	hypopharynx
27	m	50	4	2	0	2	hypopharynx
28	m	49	4	0	0	3	larynx
29	m	43	4	2	0	3	hypopharynx
30	m	53	x	2	0	x	CUP

bromide. Bands were visualized under UV light and documented with polaroid photography. The expected band size was 788 bp for actin, 485 bp for MMP2, 784 bp for MMP7, 638 bp for MMP9, 490 bp for MMP13, 551 bp for TIMP1, 427 bp for TIMP2 and 455 bp for TIMP3. The amplified bands were compared to a DNA size standard to verify bands of correct size. The relative mRNA expression was evaluated semi-quantitatively by calculating the ratio of specific MMP or TIMP signals to the signal of beta-actin that arbitrarily was set to 100% (0%=not detectable, 25%=signal weakly detectable, 50%=detectable signal, but weaker than actin, 100%=as strong as actin signal, 150%=signal slightly higher than actin, 200%=signal clearly higher than actin).

Statistics. Data correlation was examined with the tau-b-test according to Kendall and the rho-test as described by Spearman. The SPSS 11.2 software package was used for data calculation.

Results

MMP and TIMP expression. The MMP and TIMP expression patterns of the tumor samples are shown in Table III. MMP13 had the highest of all mean values at 113.54%. Table IV summarizes the quantity and frequency of the detected RNA signals of MMPs and TIMPs in the specimens excised from metastases (n=6). In the metastasis group, the MMP signals exhibited a different distribution compared to the primary tumor group. Also in the metastasis group, MMP13 was still the most frequently occurring MMP (100% of the specimens *versus* 96% in primary tumors), with a mean value of 83.33% *versus* 113.54%. However, it was not expressed most strongly. In metastases, the strongest signal was that of MMP9 (mean value of 100%), while the standard deviation of 28 indicates a very broad variability within the RNA quantity. MMP2 was described in half of all cases (50%), the mean value being about 20%. The missing signal of MMP7 was very uniform, not being detected in any of the examined metastases.

Patterns and correlations of MMP expressions. Established recurring expression patterns within the MMPs could not be described with ultimate certainty in the examined specimens. The expression of a specific MMP was not related to the expression of other MMPs. In comparison to that, the three TIMPs were found together in 100% of the tumor specimens. The signal intensity of TIMP2 and -3 showed a statistically significant positive correlation [correlation coefficient: Kendall-tau-b 0.324 (p=0.036), Spearman-rho 0.327 (p=0.039)]. A high TIMP2 signal was associated with increased TIMP3 levels. However, no such correlation could be detected for TIMP1. Correlations also were found between the RNA quantity of single TIMPs and MMPs. The primary tumor specimens alone showed a significant negative correlation between MMP7 and TIMP2 [correlation coefficient: Spearman-rho –0.353 (p=0.045), Kendall-tau-b –0.318 (p=0.045)]. This means that lower values of MMP7 RNA were associated with high values for TIMP2 and *vice versa*. In the case of metastases, the RNA quantities of MMP13 correlated positively with TIMP2 [correlation coefficient: Kendall-tau-b 0.727 (p=0.037), Spearman-rho 0.850 (p=0.016)].

Correlations between MMPs and clinicopathological factors. The correlation between the lymph node status and the quantity of expressed MMP13 RNA was negative, *i.e.* the quantity of MMP13 decreased with an increased number of metastatically-affected lymph nodes [Kendall-tau-b –0.404 (p=0.016), Spearman-rho –0.448 (p=0.014)]. Also, the quantity of MMP13 RNA and the histological grading G correlated negatively [Kendall-tau-b –0.291 (p=0.049), Spearman-rho –0.333 (p=0.048)]. The systemic extension of the tumor disease was associated with an increased quantity of detectable MMP9 RNA [Kendall-tau-b 0.341 (p=0.025), Spearman-rho 0.377 (p=0.024)]. No statistically significant correlation between the primary tumor size T and MMP or TIMP expressions could be found.

Table II. *Primer sequences.*

Name	Orientation	Sequence
beta-Actin	sense	**5'**-GATGATGATATCGCCGCGCTCGTCGTC-**3'**
	antisense	**5'**-GTGCCTCAGGGCAGCGGAACCGCTCA-**3'**
MMP2	sense	**5'**-CCACGTGACAAGCCCATGGGGCCCC-**3'**
	antisense	**5'**-GCAAGCCTAGCCAGTCGGATTTGATG-**3'**
MMP7	sense	**5'**-CCTGGCAGCCTGGCCCTGCCGCTGCCT-**3'**
	antisense	**5'**-AATGAATGGATGTTCTGCCTGAAGTTTC-**3'**
MMP9	sense	**5'**-GGTCCCCCCACTGCTGGCCCTTCTACGGCC-**3'**
	antisense	**5'**-GTCCTCAGGGCTCTGCAGAATGTCATAGGT-**3'**
MMP13	sense	**5'**-GACTTCACGATGGCATTGCTG-**3'**
	antisense	**5'**-GCATCAACCTGCTGAGGATGC-**3'**
TIMP1	sense	**5'**-TGCACCTGTTGTCCCACCCCACCCACAGACG-**3'**
	antisense	**5'**-GGCTATCTGGGACCGCAGGGACTGCCAGGT-**3'**
TIMP2	sense	**5'**-AAGGAAGTGGACTCTGGAAACG-**3'**
	antisense	**5'**-TTGATGCAGGCGAAGAACTTGG-**3'**
TIMP3	sense	**5'**-ATCAAGTCCTGCTACTACCTGC-**3'**
	antisense	**5'**-TCATTCTTTCTGGCATGGCACC-**3'**

Table III. *MMP and TIMP levels in primary HNSCC tumors.*

Name	Positive samples	Positive of total (%)	Mean (%)	SD	Median
MMP2	18	75	25.00	3.69	25
MMP7	16	67	48.96	12.27	25
MMP9	18	75	54.17	11.13	50
MMP13	23	96	113.54	11.75	100
TIMP1	24	100	101.04	6.81	100
TIMP2	24	100	79.17	5.14	100
TIMP3	24	100	42.71	2.37	50

Table IV. *MMP and TIMP levels in HNSCC metastases.*

Name	Positive samples	Positive of total (%)	Mean (%)	SD	Median
MMP2	3	50	20.83	10.04	12.50
MMP7	0	0	0.00	0.00	0.00
MMP9	5	83	100.00	28.87	100.00
MMP13	6	100	83.33	16.67	75.00
TIMP1	6	100	100.00	0.00	100.00
TIMP2	6	100	83.33	16.67	75.00
TIMP3	6	100	54.17	10.04	50.00

Expression of MMPs in different tumor locations. The location of the primary tumor was not signification for the expression patterns of MMPs and TIMPs in the current specimens. No significant differences were found in the RNA expression levels in tumors of the larynx, oropharynx and hypopharynx.

Expression of MMPs in cell lines. Table V summarizes the expression patterns of the tested tumor cell lines. MMP9 exhibited a signal level of 25% in the keratinocyte cell line, whereas the other tested MMPs, -2, -7 and -13 were not detected. In contrast, all three TIMPs could be detected, the signal intensity of TIMP1 reaching 100%, and that of TIMP2, –3.50%.

Table V. *Statistical description of the signals of MMPs and TIMPs in tumor cell lines.*

Name	Positive samples	Positive of total (%)	Mean (%)	SD	Median
MMP2	3	43	10.71	13.36	0
MMP7	5	71	53.57	44.32	25
MMP9	3	43	17.86	18.90	25
MMP13	6	86	64.29	37.80	50
TIMP1	7	100	100.00	0.00	100
TIMP2	7	100	85.71	24.40	100
TIMP3	6	86	35.71	19.67	50

Correlations between MMPs and clinicopathological parameters of the examined cell lines. Certain expression patterns showing correlations between the single parameters could also be found in the examined cell lines. There was a negative correlation between the lymph node status of the original tissue of the cell lines and the quantity of expressed MMP13 RNA [Kendall-tau-b –0.653 ($p=0.047$), Spearman-rho –0.683 ($p=0.045$)]. Within the detected quantities of the single

MMPs and TIMPs, a positive correlation could be detected between MMP2 and TIMP3 [Kendall-tau-b 0.694 ($p=0.038$); Spearman-rho 0.725 ($p=0.0339$)].

Discussion

According to Liotta *et al.* (10), tumor cells must possess three properties to be locally invasive and lead to metastasis: (i) the ability to degrade components of the extracellular matrix (ECM), (ii) to attach to and detach from proteins of the ECM and (iii) to move through small defects in the matrix. The angiogenic potential of tumor cells is important for the metastatic process, too. However, these are not characteristics of malignant cells since benign neoplasms can also induce growth of new vessels, as observed in angiomas. Numerous studies have emphasized the significance of MMPs and TIMPs. The proteolytic ability of MMPs not only plays a major role in ECM remodeling, but also affects adhesion and mobility of tumor cells (11) and modulates angiogenic substances such as VEGF (12). The four TIMP family members are characterized by several common structural features and are found widely in most human tissues and body liquids (13-16). Next to their ability to inhibit MMPs, TIMPs also possess several other biological features. While TIMPs -1 and -2 are regularly found in a soluble form, TIMP3 remains bound to the ECM (14). All members of the MMP family have the ability to break down the ECM and thus remove physical barriers for the spread of malignant cells. However, the ECM is dynamic and continuously changing. The structural proteins, growth factors and latent enzymes of the matrix have an impact on the surrounding cells as well as cell-cell interactions. Under physiological conditions, MMPs contribute to the maintenance of the ECM by degrading unwanted proteins. Destruction of the ECM by degradation of its structural components is a prerequisite for invasion and metastasis, specifically degradation of basal membrane constituents such as collagen IV (10). The aim of the present study was to answer basic questions, such as the level of MMP-mRNA by using RT-PCR. The level of MMP activity was not considered. Also, it has to be pointed out that, in contrast to histological studies, RT-PCR does not provide information on MMP distribution in the tested tissues. In this study, MMP2 was found in 75% of primary tumors and 50% of the metastases, respectively. The expression neither depended on the presence of other MMPs, nor did it correlate with clinical parameters such as lymph node and distant metastatic status or grade of differentiation. However, there were tendencies between MMP2 levels, tumor stage (T) and MMP7 and -9 levels. Despite a tendency for higher MMP2 expression levels in tumors that behave more invasively, there was no clear correlation with the metastatic behavior. MMP9 RNA was detected in 75% of primary tumors and 83% of metastases. Next to a potential correlation of MMP2 and -9 levels, the latter also correlated with RNA quantity and tumor stage (T). Higher MMP9 levels were found in tumors of higher stages. Also, a correlation was found between distant metastatic status (M) and quantity of MMP9. MMP9 appears to be associated with higher invasion and metastatic spread, since it was found to be more highly expressed in distant metastases. A tendency to higher MMP9 expression levels, as observed in the present study, is consistent with other published results (17, 18), whereas there are no reports of a negative correlation between MMP2 expression and tumor T-stage. In the present study, MMP13 was detected in primary tumors as well as metastases of nearly all the specimens. The MMP13 expression levels tended to increase with higher T-stage. However, there was a negative correlation with lymph node status (N), since lymph nodes affected by tumor tissue led to a statistically significant decrease of the MMP13 message. This corresponds to reports of other groups (19-21). In summary, it can be concluded that MMP13 plays an important role in malignancies of the upper aerodigestive tract. One potential explanation regarding the observed decrease of the MMP13 signal in less differentiated tumors could be that MMP13 is characteristic of the original benign tissue and seems to decrease with loss of differentiation. Since MMP13, in general, was found regularly in most tested tumor specimens, it might be useful as a marker of this disease entity. There are few reports about MMP7 in carcinomas of the upper aerodigestive tract (22, 23), most of them pointing to a contribution of MMP7 in carcinoma progression. In the present study, MMP7 was found in the majority of primary tumors (67%), however in none of the tested metastases. There was no correlation of MMP7 level with tumor, lymph node, metastatic stage or level of differentiation, except for a tendency of this MMP to decrease in higher tumor stages. Therefore, no clear prognostic role can be attributed to MMP7 in carcinomas of the upper aerodigestive tract. The TIMP expression pattern in carcinomas of the upper aerodigestive tract is regularly described in the context of MMP expression. Publications considering TIMPs alone are relatively rare. Heissenberg *et al.* (5) published a detailed review on TIMPs -1 and -2. They found TIMP1 in nearly all examined primary tumors. In the specimens of patients developing recurrences in the further course of the disease, no TIMP1 was found, however, TIMP2 was nearly always detected. The whole group of primaries expressed MMP9 only in some specimens. Heissenberg *et al.* (5) did not find TIMP1 in any of their examined cases. They postulated, that the absence of TIMP1, particularly in the presence of MMP9, indicates a higher aggressiveness of the tumor. However, Kurahara *et al.* (24) observed contrary findings. Primary tumors with metastatic spread revealed significantly higher TIMP1 levels

than tumors without metastatic spread, whereas TIMP2 did not correlate with metastatic spread. They concluded that increased MMP expression is more important for metastatic spread than a decrease of inhibiting factors such as TIMPs. Similarly, Xu *et al.* (25) were able to detect higher TIMP1 values in laryngeal than in pharyngeal carcinomas. Also, they observed on average higher TIMP1 expression levels compared to TIMP2, while Heissenberg *et al.* (5) described opposite results. Charous *et al.* (26) could not detect differences between TIMP1 expression levels of primary tumors and metastases, whereas tumors in advanced T-stages were found to tend to lower TIMP1 levels. Interestingly, the present study found a negative correlation between TIMP2 and MMP7, *i.e.* high TIMP2 levels were associated with low or not-detectable MMP7 expression. Additionaly, the quantity of TIMP2 correlated positively with TIMP3. This could be explained, for example, due to mutually preferred substrates for inhibition. Not many studies have investigated TIMPs -2 and -3 in head and neck cancer. The present study, however, implicated these TIMPs in head and neck cancer development. Riedel *et al.* (27) did not find any correlation between MMP9 expression and tumor location. An extensive MMP study was published by O-Charoenrat *et al.* (28). Here, the authors could not find a correlation between MMP expression and tumor location. However, correlations were detected between MMPs -1, -9, TIMP1 and advanced tumor stage, as well as between the expressions of MMPs -2, -7, -9, -11 and the lymph node status of tumors. The extent of the study by O-Charoenrat *et al.* (28) shows first approaches to detect such expression patterns. For example, tumors with lymph node metastases frequently had more MMPs expressed, however, no clear MMPs expression pattern could be deducted. Further studies will be required to reveal more about the potential interplay of different MMPs and their role in HNSCC progression. In this study, the MMP and TIMP expression patterns of HNSCC cell lines and primary tumor specimens was similar. All tested MMPs and TIMPs were regularly found in the specimens, with MMP13 and, to a lesser extent, MMP7 being the most prominent proteases. MMP9 and -2 levels were found to be lower in tumor tissues. TIMP1 was regularly expressed in all cell lines, whereas TIMPs -2 and -3 had a somewhat lower expression. One major purpose of previous studies, as well as this present study, has been to discover prognostic markers for carcinomas of the upper aerodigestive tract, thereby enabling the clinician to assess the metastatic potential of HNSCC tumors. Due to their implication in tumor progression and metastatic spread, MMPs are today the focus of many studies. Until now, however, it was not possible to establish prognostically relevant MMP markers, despite solitary reports of single groups. Unfortunately these results do not show any standardization, so that a final conclusion often remains impossible. Therefore, it has to be the goal of future investigations to standardize the methods of MMP and TIMP detection and perform multicenter studies. This would greatly help in the correct recognition and discovery of HNSCC-relevant MMPs and TIMPs.

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